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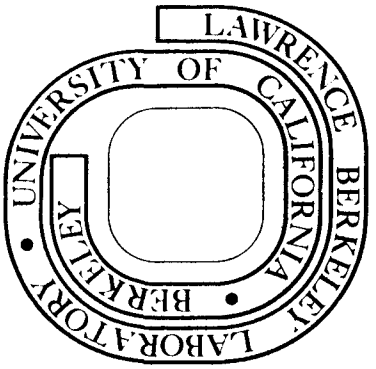

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MEASUREMENT OF VERY SHORT FLUORESCENCE LIFETIMES BY SINGLE PHOTON COUNTING

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ABSTRACT

Measurement of very short fluorescence lifetimes by the single photon technique is made possible by an improved fluorescence lifetime system. Fluorescence lifetimes of 4.94 $\pm$ 0.07 nsec for anthracene in cyclohexane, 640 $\pm$ 30 psec for diphenylbutadiene in cyclohexane and 90 $\pm$ 30 psec for erythrosin in water were determined. The use of a small wavelength shift between excitation and emission minimizes the effect of the wavelength dependence of the photomultiplier response and light pulser emission. The effects of de-aeration of solutions and time averaging of the excitation spectrum are presented. We investigated the origin of small amplitude early and late artifactual peaks in the light pulser and fluorescence spectra. Complications in the analysis of lifetime data introduced by intrinsic fluorescence and phosphorescence processes in commonly used absorption filters are discussed. Certain "blind spots" are found in the electronic pulse pileup rejection schemes most commonly used in photon counting.

INTRODUCTION

Fluorescence lifetime measurements are commonly performed using single photon counting systems,[1,5] phase shift instruments,[22,23] or mode-locked laser systems.[17,19] Phase shift instruments provide good speed and accuracy but they offer limited capabilities for the resolution of multiple component spectra. Laser systems provide time resolution in the picosecond time domain but suffer from poor signal to noise ratios and diminished multiple component capabilities. Photon counting systems operate with excellent signal to noise ratios which result in a wide dynamic range in the time and intensity intervals over which the fluorescence can be recorded. This wide dynamic range makes possible an accurate analysis of multiple component fluorescence data. To date, however, the single photon technique has provided a slower time resolution than either other technique. The measurements reported in this paper represent an extension of the single photon technique into the very short lifetime domain. In this short time domain, the inherent wider dynamic range of photon counting provides important advantages in measurements on chemical and biological systems where highly quenched fluorescence is observed from multiple chemical species or physical environments. In addition, this increased time resolution can improve the accuracy of multiple or single component lifetime determinations in longer time domains.

THE INSTRUMENT

Methods and Materials

The design of the single photon counting lifetime system is described in reference 24. For all measurements reported in this paper the RCA 8850 photomultiplier was used at an operating temperature of 0°C and a supply voltage of 3000 V. All fluorescence lifetime measurements were performed with the samples at room temperature. The time axis for the multichannel

analyzer was calibrated from peak shifts induced by calibrated length delay cables. The light pulser was operated in air at 1 atmosphere. The light pulser voltage was 6,000-9,000 V which yielded a detected flash rate of 13,000-26,000 flashes per second. The data collection rate [(time-to-amplitude-converter rate)/(light pulser flash rate)] was 10% or less for all measurements.

The excitation and emission wavelengths were selected by optical filters. The filters used in each experiment were: Erythrosin -- Optical Industries 4900 Å and 5500 Å interference filters; Diphenyl Butadiene -- Corning 3-72 and 3-74 in series for emission, Optics Technology 330 nm interference filter for excitation; Anthracene -- Corning 3-72 and 3-74 in series for emission, Kodak 18A for excitation. The diaphragm was adjusted to an opening of 2 mm to 20 mm diameter in these studies. The light pulser profile for the anthracene and diphenyl butadiene experiments was recorded using a polished metal front surface reflector from a Perkin-Elmer MPF3L fluorescence spectrometer. The light pulser profile for the erythrosin experiment was obtained using distilled water in a 1 cm x 1 cm fluorescence cuvette as the scatterer. Additional details are provided in the experimental section for each compound investigated.

Filters

Colored glass or interference filters are used in our system to isolate the proper wavelengths of excitation and fluorescence emission for each sample. Filters permit recordings with high collection efficiency and with a broad bandpass, which increases the system sensitivity. Glass filters also introduce significant fluorescence and phosphorescence artifacts into lifetime measurements. Exposure of colored glass filters to bright room lights introduces phosphorescence emission which can be detected by the photomultiplier as an increase in the background count rate for over 1 hour after light exposure. In addition, the filters capture scattered or incident exciting light and fluorescence photons and convert them into filter fluorescence

arising in the glass. Filter fluorescence can add a low intensity ($\sim$ 0.5%) lifetime component to each spectrum taken. This system artifact has a significant effect on lifetime measurements, especially when the filter and sample lifetimes are very different. Examples and discussion of this problem appear in the Experimental section of this paper.

Pulse pileup rejection

The single photon counting technique of lifetime measurement is based upon the concept that the probability distribution for the emission of a single photon of fluorescence following a single exciting light pulse is identical to the intensity-time profile of the cascade of all the photons which are emitted following a single flash of exciting light. The single photon emission probability distribution is built up by repetitive exposure of the sample to short bursts of exciting light and recording of the time of arrival of the first photon of fluorescence following each exciting pulse. If more than one fluorescence photon arrives at the photomultiplier following a single excitation flash, only the first photon will be recorded because only one fluorescence photon stop pulse can be processed at the time-to-amplitude-converter per excitation start pulse. The loss of later fluorescence events will distort the recording of the probability distribution by artificially enhancing the early portion of the fluorescence spectrum. To avoid this problem, several techniques for the prevention of this pulse pileup have been developed.

The simplest method for the prevention of multiple photon events is to attenuate the light intensity reaching the photomultiplier so that multiple photon events are extremely rare. Under these circumstances, the multiple photon contribution to the spectrum will result in a distortion of only a few percent in the data, which can usually be tolerated. The limited rate of data collection associated with this

approach increases the time required to record a spectrum. This can be an important limitation when labile biological samples are under investigation. There is a theoretical correction for multiple photon events which allows higher data collection rates and shortens the recording time.[2,3,4] Certain experimental uncertainties make the correction difficult to apply accurately, however.[3]

Pulse pileup rejection can also be accomplished electronically by utilizing a digital inhibit circuit. If the output of the time to pulse height converter is delayed by a suitable length of cable, then a pulse counter can be used to gate the input of the analogue to digital converter. The pulse counter will inhibit storage of the fluorescence event if more than one photon of fluorescence is detected following a single lamp flash.[5,3] With this technique, data collection rates [(time to amplitude converter rate)/(light pulser flash rate)] of up to 20% can be utilized.[3] It is important to note, however, that this method suffers from a frequently overlooked 'blind spot'. The discriminators used in our lifetime system and in other systems require a reset time of at least 50 nanoseconds before they are able to process a second fluorescence photon. During this period the pulse counter will fail to detect the arrival of subsequent photons. Since the probability for photon emission peaks sharply and falls off exponentially or as a sum of exponentials (for short light pulser flashes), if multiple photon events occur, they will most likely occur with a small time separation. This method will be blind to precisely those multiple photon events which are most likely to occur, especially when short fluorescence lifetimes are being measured.

An alternative electronic pulse pileup rejection scheme is available. If two photons are captured by the photomultiplier within a very short time interval, then their resulting photoelectron cascades will overlap. This overlap will cause the amplitude of the photomultiplier output

pulse to be outside the normal range of single photon output pulse heights. An upper limit threshold can then be built into the photomultiplier's discriminator in order to reject all pulses of large amplitude, originating from multiple photon events. The excellent multiple photon resolution of the RCA 8850 lends itself to this mode of pileup rejection.[6,27] We have incorporated this method of pulse pileup rejection in our lifetime system.

This method also has its characteristic 'blind spot'. If the separation in time between photons is much larger than the FWHM of the output signal of the photomultiplier, then the electron cascades will be resolved from each other and the amplitude of the output pulse will not be significantly different than for single photon events, although two separate peaks will be seen. In this case, the pulses will pass through the discriminator window but only the first photon event will be recorded in the analyzer. This problem will be particularly severe when long fluorescence lifetimes are measured, since the multiple photons are more likely to be separated by longer time intervals. Most single photon counting photomultipliers exhibit a FWHM output of 2-3 nsec in response to a single photon event. Thus, multiple photon events will not be detected by this pulse pileup method if the two photons are separated by more than approximately 2 nsec.

We have seen that pileup rejection based on a digital inhibit circuit exhibits a blind spot if the photons are separated by less than about 50 nsec. Pileup rejection based on discriminator windows is ineffective for photons separated by more than approximately 2 nsec. Even a combination of these electronic methods will be unable to detect all multiple photon events. A modified discriminator window method of pulse pileup rejection has been designed, however, which can effectively record all multiple photon events.[6] This method utilizes a single channel

analyzer with upper and lower thresholds and an appropriately slow time constant to stretch the input pulses from an intermediate dynode of the photomultiplier. Events occuring within the measuring time interval of the system will overlap and the window in the single channel analyzer can select for single photon events. The analyzer produces a gating pulse that inhibits recording of the photon event whenever multiple photons are detected.

It is important to be aware of these deficiencies of pulse pileup rejection when making lifetime measurements If the simple electronic pulse pileup rejection system is chosen, it should be supplemented by an attenuation of the data collection rate and/or the application of appropriate theoretical corrections for multiple photon events. In our lifetime system, the simple discriminator based pileup rejection system is supplemented by an attenuation of the TAC count rate to 10% or less of the lamp firing rate. This method was used for all measurements reported in this paper.

Early and late spectral peaks

All published excitation and fluorescence spectra from single photon counting instruments exhibit secondary peaks of low intensity which occur before and after the primary peak.[1,6,7] If the photomultiplier dynode string is poorly adjusted, then further peaks will appear.[7] An expanded profile of the light pulser flash as measured on our instrument can be seen in Figure 1. Both the early and late peaks exhibit approximately 0.35% of the intensity of the primary peak. The early peak occurs 7 nsec before the main peak, the late peak occurs 16 nsec after the main peak. It has been proposed that these supplemental peaks arise from internal processes in the photomultiplier.[1,8] Our findings confirm this inter-pretation. The peaks do not arise from light reflections in the sample chamber because the

profile is unchanged if a light pipe is used to channel light pulser flashes directly into the photomultiplier. The peaks are not a function of the light source because they are also seen in the record of the flash profile using a light emitting diode as the emission source. The intensity of the early peak is, however, sensitive to the area of illumination of the photomultiplier. In Figure 1, we observe that restricting the illumination to the center of the photocathode causes the early peak to increase in intensity while the late peak is unaffected. The dynode structure underlies the center of the photocathode and is preferentially illuminated in this case. Apparently, the early peak arises from photons which are not absorbed by the photocathode, but rather pass through this surface and directly strike the first dynode causing an early electron cascade. The origin of the late peak is uncertain. It appears to be an internal electron reflection of some sort since it is unaffected by variations in the illumination of the photocathode. One possibility is that electrons from the first dynode travel back up to the photocathode, initiating a late starting secondary electron cascade.[8]

If excitation and fluorescence spectra are not recorded under identical conditions, the amplitudes of the early and late peaks can differ between spectra. These differences can lead to problems when a deconvolution analysis of the lifetimes is attempted. The variation in early peak amplitude can be minimized if the emitting volumes of the samples used for excitation and fluorescence spectra are equalized. Equalization of emitting volumes will also reduce variations in photo-multiplier response characteristics associated with illumination of different photocathode areas.[7]

DATA ANALYSIS

The fluorescence emission from a set of identical fluorophores in response to a delta pulse of exciting light is given by the relation:

$$f(t) = \sum_{i-1}^{N} \alpha_i e^{-t/\tau_i}$$

where N is the number of fluorescence components, and α_i and τ_i are the relative intensity and lifetime respectively of the i^{th} component. The experimentor must determine the best fit values for α_i, τ_i and N for each sample investigated. Whenever the exciting light pulse has an appreciable time width in comparison to the fluorescence signal, a convolution integral must be solved in order to extract the lifetime parameters.[25] A number of theoretical approaches have been developed to extract the amplitude and lifetime values from the experimentally determined excitation and fluorescence profiles.[9,10,11] We have utilized the method of moments technique[12,13] for all data analysis reported in this paper.

EXPERIMENTAL

Anthracene in cyclohexane

To verify the accuracy of the overall lifetime system we have chosen to investigate pure compounds with well established fluorescence life-times. Anthracene is commercially available in high purity and there is agreement in the literature on its fluorescence lifetime in cyclohexane. Values of 4.9 nsec[15] and 5.0 nsec[16] have been reported. The fluorescence of deoxygenated anthracene in cyclohexane is shown in Figure 2. The sample consists of 0.3 mg/ml anthracene (99.99 mol%, Materials Ltd., Inglewood, N.J.) in cyclohexane (Chromatoquality 99+ mol%, M.C.B., Norwood, Ohio) which is deoxygenated by bubbling with nitrogen gas for 15 min inside a nitrogen-filled glove box. The best-fit analysis, shown in Figure 2, yields an anthracene lifetime of 4.94 nsec with a minor component $[\alpha_2/(\alpha_1 + \alpha_2) = 0.4\%]$ of 21 ns.

The minor lifetime component is seen in all spectra from a variety of compounds that we have investigated. This system artifact arises primarily from two sources. In many spectra the system artifact represents a true background system fluorescence which arises primarily from the glass absorption filters used to isolate the excitation and fluorescence light. This is the case with anthracene in cyclohexane since the same low intensity long lifetime fluorescence is observed when cyclohexane alone, or any other scatterer, serves as the sample in the setup used to record the anthracene fluorescence. An additional factor that contributes to the system artifact is the sensitivity of the lifetime analysis, especially in the tail region where long lifetime components dominate, to the size and distribution of the scattering or emission volume within the sample. In the case of excitation spectra, the use of a large diffuse scatterer such as the sample solvent in a cuvette, will yield a broader excitation spectrum with a more pronounced tail component than is obtained from a small, focussed scattering image from a polished metal surface. If the image size and intensity distribution properties are not exactly matched in the fluorescence and excitation samples, then different areas of the photomultiplier surface are illuminated and an artifactual small intensity, long lifetime fluorescence component will be observed in the analysis.

The presence of the system artifact can be demonstrated in any particular set of data by the improved fit that a multi-component lifetime analysis will yield. The method of moments program contains a number of objective fit tests which permit the experimentor to choose the best-fit analysis of his data from the one component, two component or three component analyses that the program allows. In addition, a visual examination of the experimental and calculated fluorescence curves will often reveal the presence of the small amplitude long lifetime system artifact in the data.

The measurement of a relatively long fluorescence lifetime, such

as 5 nsec for anthracene, is not seriously complicated by the presence of the system artifact. In the anthracene experiment, a one component analysis yields a value of 5.02 nsec compared to the best-fit two component value of 4.94 nsec. The removal of dissolved oxygen is, however, an important requirement. Since molecular oxygen normally exists in a triplet state, it can serve as an effective fluorescence quencher. The lifetime of anthracene in air saturated cyclohexane (without deoxygenation) was determined to be 4.0 nsec. Control of solution oxygen levels is important for accurate lifetime determinations.

Diphenyl butadiene in cyclohexane

Accurate subnanosecond lifetimes have been reported for very few compounds. One of the fastest, well characterized chemical systems is trans,trans-1,4-diphenyl-1,3-butadiene in cyclohexane. A lifetime of 0.63 nsec has been reported for this system using a quantum counter to minimize photomultiplier wavelength effects.[7] The emission of this compound measured on our lifetime system is shown in Figure 3. The sample contains 1.0×10^{-5} M trans,trans-1,4-diphenyl-1,3-butadiene (M.C.B., Norwood, Ohio; twice recrystallized from carbon tetrachloride before use) in cyclohexane (chromatoquality 99+ mol%, M.C.B.) which had been deoxygenated in a stream of nitrogen gas as described for anthracene. The best fit analysis yielded two lifetime components. The observed lifetimes were 0.64 nsec for diphenyl butadiene and 15 nsec for a minor $[\alpha_2/(\alpha_1 + \alpha_2) = 0.18]$ system artifact component. In this case the presence of the very minor level of system artifact fluorescence has a significant effect on the lifetime analysis. If the presence of this minor component is ignored, and a one component lifetime analysis is attempted, the result is an apparent lifetime of 0.96 nsec. To obtain accurate analysis of short lifetime samples, it is important to use a two component analysis whenever even minor contamination from long lifetime

sources is encountered. In the absence of multi-component capabilities in the analysis, the results may be quite inaccurate.

Erythrosin in buffer

To our knowledge, lifetime measurements on compounds with lifetimes of under 480 psec[26] have not been reported in the literature from single photon counting systems. To test the limits of our measurement capabilities, we examined the lifetime of erythrosin fluorescence. Values of 57 psec[17], 90 psec[18], and 110 psec[19] have been reported for the lifetimes of erythrosin and erythrosin B (the disodium salt of erythrosin) in water. These measurements were obtained with mode-locked laser systems. Our sample of erythrosin (Eastman, Rochester, N.Y.) was purified by passage through a silica gel-methanol column and suspended in a buffer solution of 40 mM Tricine-NaOH, 2 mM EDTA, pH 8.0. We determined the lifetime of this erythrosin solution to be 90 psec for fluorescence emission at 550 nm that is excited at 490 nm, with a minor system artifact component of 4.7 nsec, $[\alpha_2/(\alpha_1 + \alpha_2) = 0.04\%]$. The experimental and predicted lifetime curves are shown in Figure 4.

To obtain accurate measurements of very short lifetimes, certain experimental conditions must be carefully controlled. The most critical problems arise from slow drifts that occur in the light pulser time profile and from the wavelength dependence of the system response. To obtain stable, optimal performance from the air gap light pulser, we have found it necessary to flush the chamber continuously with a flow of dry air during operation, to clean the electrode tips after every 10 hours of operation and to resharpen the pointed electrode into a conical tip after significant erosion has occurred. The remaining drift which occurs in the excitation profile can be minimized by recording excitation and emission profiles alternately.[20] We find that drift problems in our system are significantly decreased by recording half the excitation profile before the fluorescence measurement and half afterwards.

In the case of erythrosin, we recorded the fluorescence response for one hour. An excitation profile recorded only before the fluorescence yields a lifetime that differs by up to 40% from the value obtained from an averaged excitation profile.

The presence of a significant wavelength dependence in the system response function can complicate lifetime measurements.[9,21] This dependence arises in part in the photomultiplier, from the dependence of the kinetic energy of the photoelectrons on the wavelength of the incident photons.[21] If the fluorescence of a sample is observed at a constant emission wavelength as the excitation wavelength is progressively blue shifted, then the peak of the fluorescence response time profile remains at a constant position, but the peak of excitation profile is increasingly shifted towards earlier times. When very short lifetimes are measured from excitation and emission profiles recorded at widely separated wavelengths, this artifactual peak shift leads to the indication of an anomolously long fluorescence lifetime in the analysis. For example, if purified erythrosin in buffer is excited near 360 nm and the fluorescence is recorded at 550 nm a lifetime of 470 psec is indicated, with a poor fit of the observed and calculated fluorescence curves. If the same sample is excited at 490 nm and fluorescence recorded at 550 nm, then a lifetime of 90 psec is observed.

The presence of a wavelength dependence in the light pulser emission profile also contributes to the system wavelength dependence. The fluorescence emission from a compound whose lifetime is much shorter than the FWHM of the excitation light pulse closely resembles the excitation profile, as can be seen from an examination of the convolution integral. If a series of fluorescence profiles are recorded from a short-lifetime sample at a constant emission wavelength as the wavelength of excitation is varied, then systematic variations occur in the emission profile which arise solely from the wavelength dependence of the light pulser emission. In this manner, the wavelength

dependence of the light pulser emission is revealed, independent of any contributions from the wavelength dependence of the photomultiplier response. Using this method, we have observed that the red emission from the air gap discharge is slightly broader in time (10% broader at 490 nm than at 360 nm) and has a significantly larger tail component than the ultra-violet emission. In our judgement, the best way to minimize the system wavelength dependence, which arises from both photomultiplier and lamp effects, is to use the same light pulser filter in recording both the excitation and fluorescence profiles and to minimize the difference between the excitation and emission wavelengths.

Accuracy

Estimation of the accuracy of a lifetime determination is a complex problem. A number of parameters must be considered individually in the error estimate for each specific experiment. The spread in the light pulser profile, the single photoelectron time spread of the photomultiplier, the timing error introduced by the discrimination and the stability of the measuring system over the time span of an experiment contribute to the instrumental experimental uncertainty. In addition, the lifetime value is determined by an iterative computer de-convolution whose convergence is somewhat sensitive to the input parameters in the program. The time axis calibration for the multi-channel analyzer can provide another source of error. Other factors in the error determination are common to all experimentation, such as repeatability of the experiment, and sensitivity of the analysis to slight, reasonable variations in the experimental conditions (such as the choice of excitation and emission filters). In each experiment, the primary source of error must be identified and estimated.

In our anthracene studies, the largest source of error seems to be drifts in the system electronics which can be observed in the repeatability of the calibration of the analyzer time axis. The calibration of the time axis can

be repeated to an accuracy of approximately 1.5%. For this reason we estimate the uncertainty in the 4.94 nsec lifetime determination to be ± 0.07 nsec. In the erythrosin and diphenyl butadiene experiments, the major error factor seems to be the uncertainty in the computer determination of lifetime. In these cases, the FWHM of the lamp spectrum is equal to or larger than the lifetime under investigation. As a result, the shape of the fluorescence profile is relatively insensitive to the lifetime value over a significant range, and the error between the observed and hypothesized fluorescence profiles varies little as the hypothesized lifetime is changed. In the erythrosin experiment, for example, the reliability of the 90 psec lifetime determination was tested by assigning other values to the decay constant and comparing the quality of the fit of the experimental to the hypothesized fluorescence profile. If a lifetime of 120 psec is assumed, a distinctly poorer fit of the curves is visually obvious (root mean square residual of 59 versus 35 for the 90 ps fit). Determination of a lower limit to the lifetime from visual examination of the calculated and experimental curves is difficult because the shape of the calculated fluorescence response curves is insensitive to lifetimes below 90 psec. The method of moments program[12] does, however, contain objective fit analysis parameters that demonstrate that lifetimes below approximately 60 psec provide a poorer fit to the data than the observed value (root mean square residual of 60 at 60 psec versus 35 for the 90 psec fit). For these reasons, we assign an uncertainty of ± 30 psec to the erythrosin result and a like value for diphenyl butadiene where the same considerations dominate.

Discussion

We have demonstrated that it is possible to make accurate fluorescence lifetime measurements down to 100 psec with single photon counting systems. Short lifetime measurements require a photon counting system with a very

fast time response, such as the system we have developed,[24] and attention to a number of experimental conditions. The light pulser output must be optimized by the use of clean, sharp electrodes and the stability enhanced by continuous flushing with dry air. The lifetime analysis can be further improved by time averaging of the excitation profile. The emitting volumes of the excitation and fluorescence samples must be equalized to assure the same distribution of illumination on the photocathode. If the sample-solvent system under investigation is susceptible to oxygen quenching, the solution oxygen level must be carefully controlled. Small amplitude lifetime components from a number of sources may appear as artifacts in the results. Multicomponent lifetime analyses are required to separate the sample lifetime from these system artifacts. The presence of photomultiplier and light pulser contributions to the wavelength dependence of the system response function must be considered in the design of the experiment. When these criteria are fulfilled, it is possible to extend the wide dynamic range and multicomponent advantages of single photon counting to chemical, physical and biological systems which exhibit highly quenched fluorescence emissions.

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FIGURE CAPTIONS

Figure 1. Expanded view of the lamp flash profile; the effect of the region of photomultiplier illumination on the early and late spectral peaks. The main peak in each curve is approximately 21,500 counts. A) The front surface of the RCA 8850 photomultiplier was covered by an opaque mask with a central 5 mm diameter opening. B) The entire 50 mm diameter of the front surface of the photomultiplier was exposed.

Figure 2. Fluorescence of anthracene in cyclohexane (1.7×10^{-3} M). A) Lamp excitation profile; B) Experimental and calculated fluorescence profiles; the noisy curve is the experimental fluorescence response and the smooth curve is the calculated response generated from the excitation profile best fit intensities and lifetimes: $\alpha_1 = 0.0419$, $\tau_1 = 4.94$ nsec; $\alpha_2 = 0.00016$, $\tau_2 = 21.2$ nsec.

Figure 3. Fluorescence of 1,4-diphenyl-1,3-butadiene in cyclohexane (1.0×10^{-5} M). A) Lamp excitation profile. B) Experimental and calculated fluorescence profiles; the noisy curve is the experimental fluorescence and the smooth curve is the calculated response generated from the excitation profile; best-fit intensities and lifetimes: $\alpha_1 = 0.111$, $\tau_1 = 0.642$ nsec; $\alpha_2 = 0.0002$, $\tau_2 = 14.9$ nsec.

Figure 4. Fluorescence of erythrosin (2.8×10^{-5} M) in 40 mM Tricine-NaOH, 2 mM EDTA pH 8.0. The lamp excitation profile closely resembles the fluorescence profiles and is deleted for purposes of clarity. The noisy curve is the experimental fluorescence spectrum, the smooth curve is the calculated fluorescence response generated

from the excitation profile best fit lifetimes and intensities: $\alpha_1 = 0.429$, $\tau_1 = 88$ psec; $\alpha_2 = 0.00017$, $\tau_2 = 4.71$ nsec. Note that the fit of the data to the predicted profile deviates by less than the width of the pen trace over several orders of magnitude.

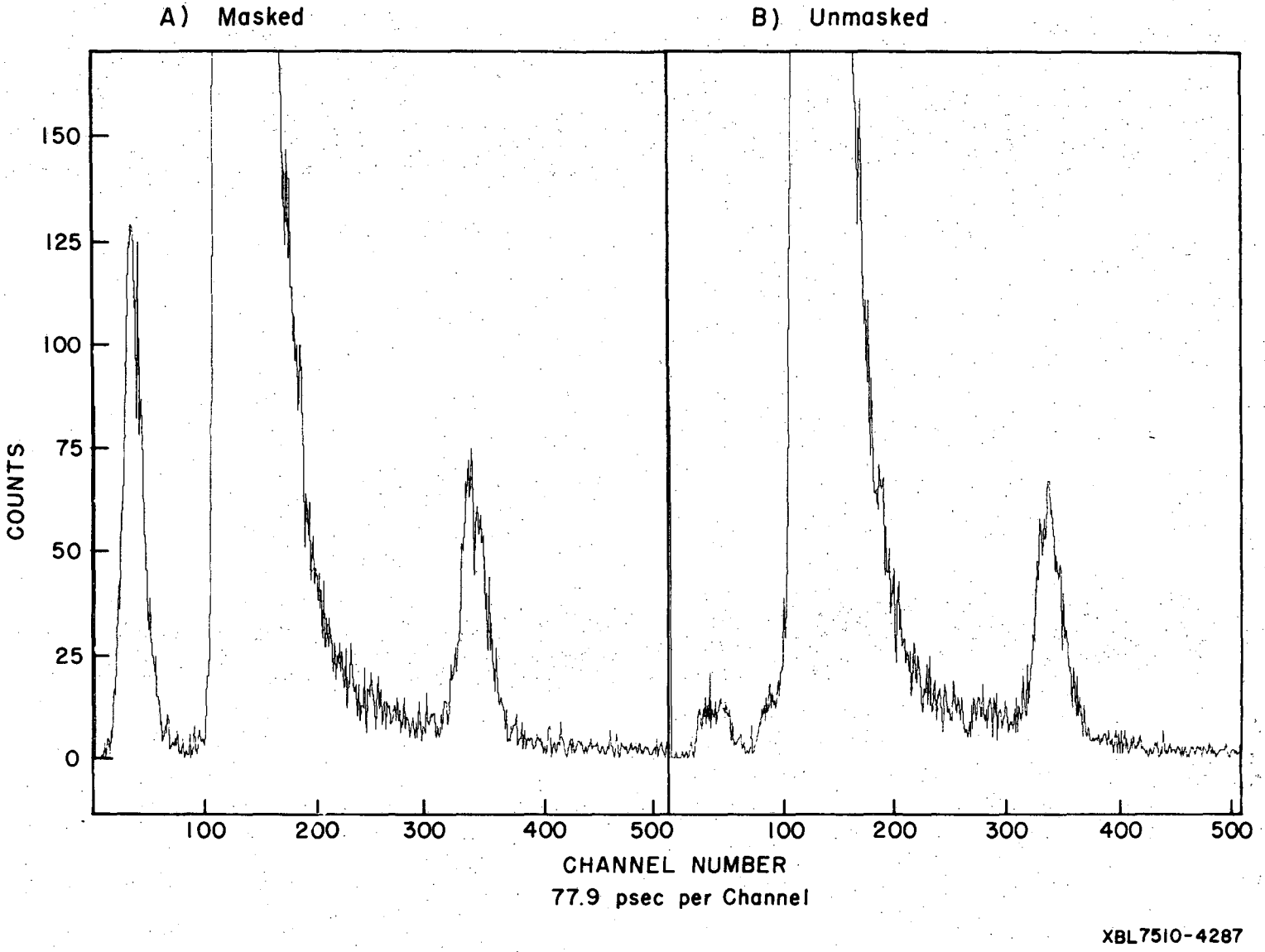
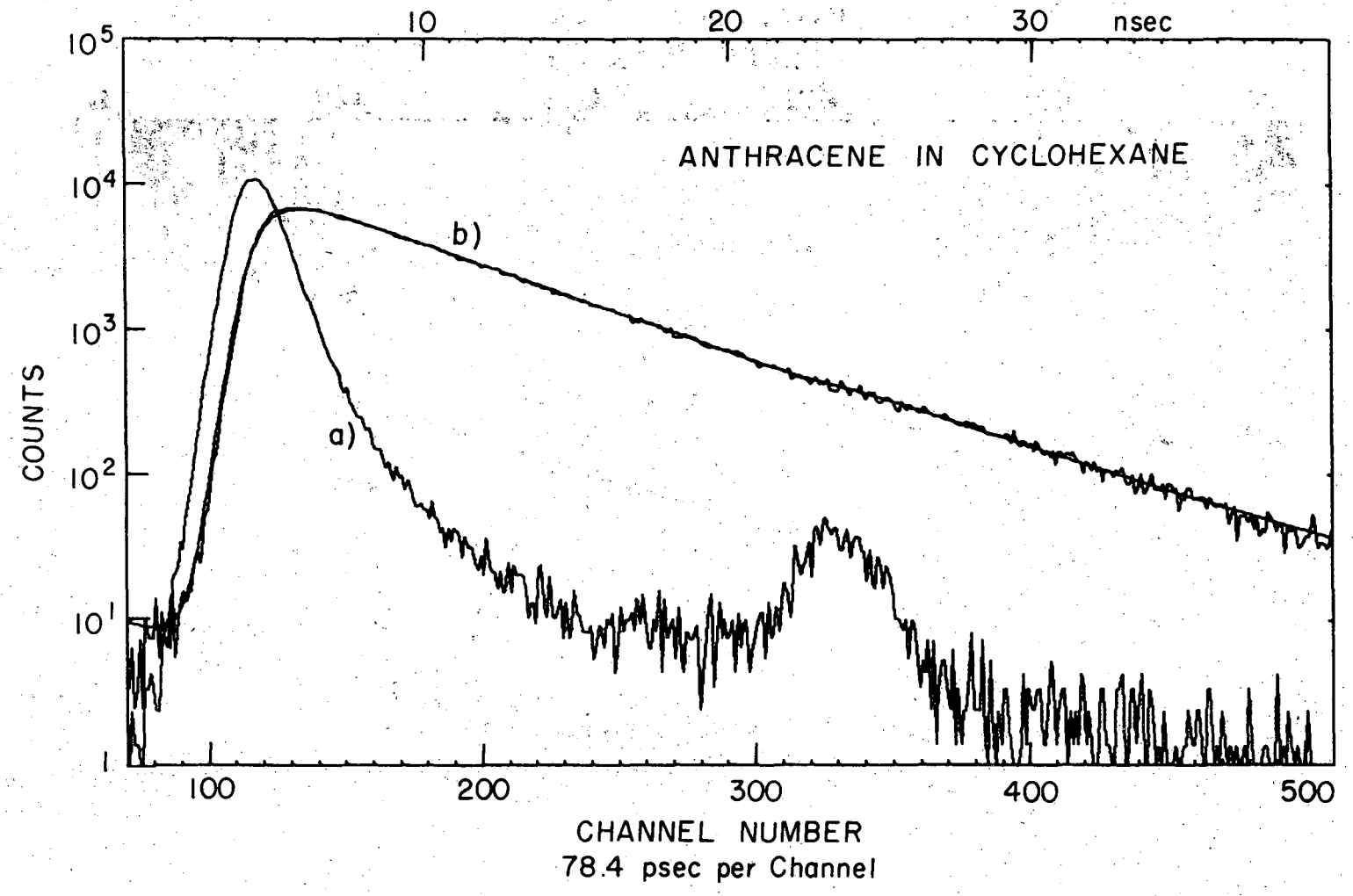

A) Masked
B) Unmasked
COUNTS
150
125
100
75
50
25
0
100
200
300
400
500
CHANNEL NUMBER
77.9 psec per Channel
XBL7510-4287

Fig. 1



XBL754-5153

Fig. 2

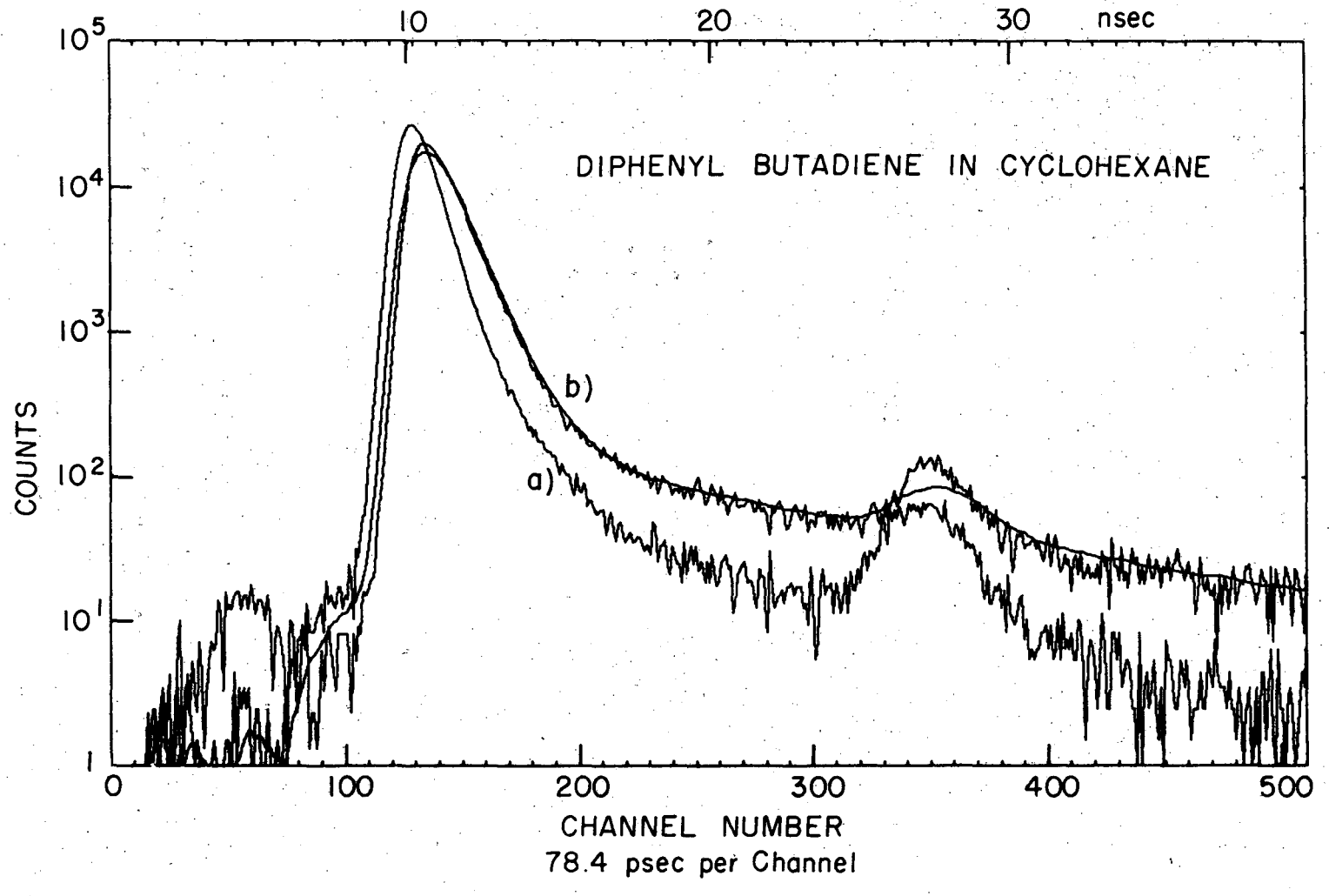

10
20
30
nsec
10^5
10^4
10^3
10^2
10^1
1
COUNTS
DIPHENYL BUTADIENE IN CYCLOHEXANE
b)
a)
0
100
200
300
400
500
CHANNEL NUMBER
78.4 psec per Channel
XBL754-5156

Fig. 3

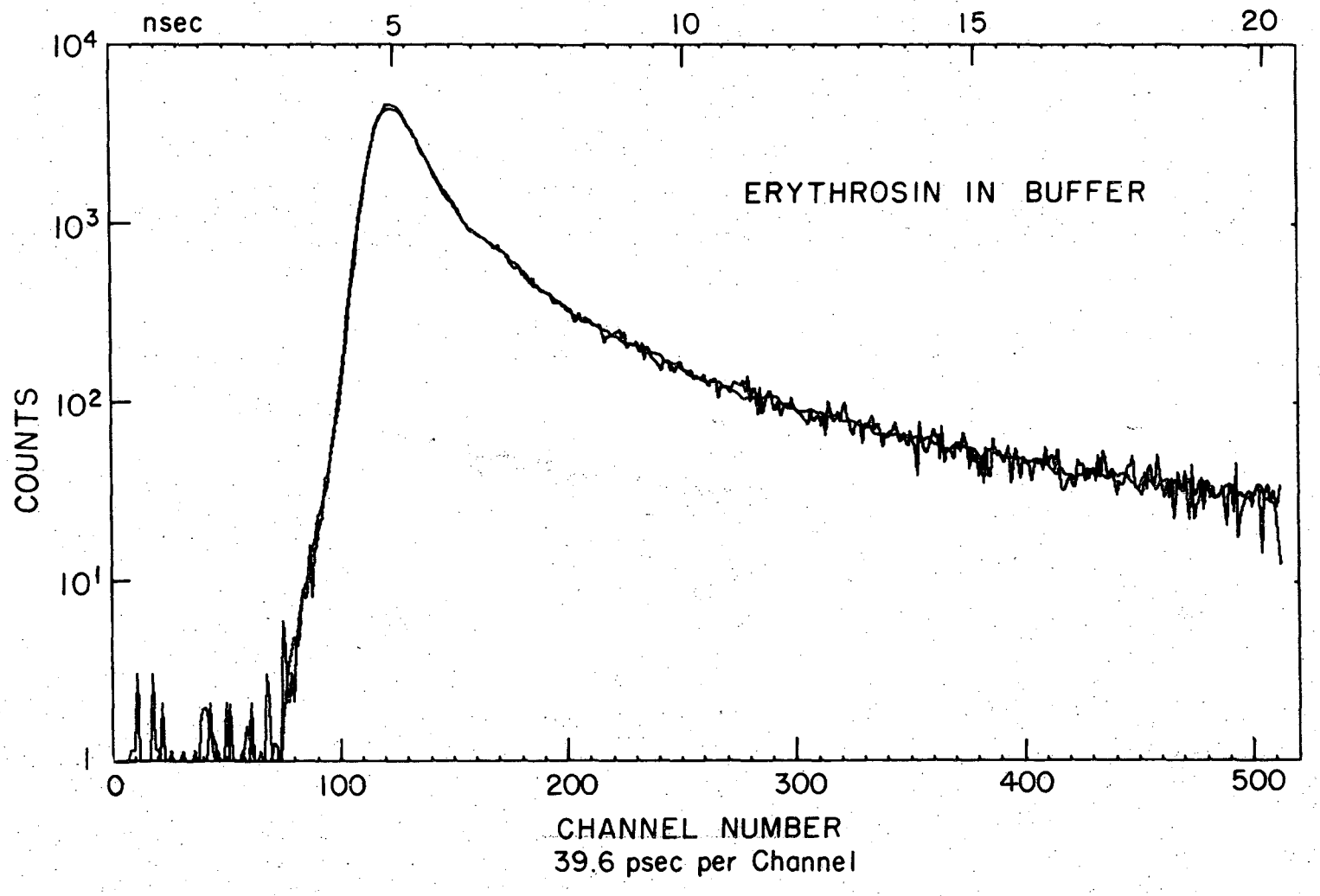


Fig. 4

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