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Simultaneous recording of the spectral, temporal and polarization properties of emission spectra

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ABSTRACT

We describe a system that records simultaneously the temporal profile of both linear polarization components of all wavelengths in an emission spectrum. Our excitation source is the vacuum ultraviolet storage ring of the National Synchrotron Light Source at Brookhaven National Laboratory, which provides a continuous spectrum of ultraviolet, visible and near-infrared light consisting of ≈ 1 ns FWHM pulses at a repetition rate of ≈ 50 MHz, and with identical temporal profiles at all wavelengths, although any source with similar temporal properties could be used. A single excitation band is selected by a monochromator and linearly polarized before reaching the sample. Fluorescence can be monitored either along an axis perpendicular to the excitation beam, or at near normal incidence. A polarizer divides the fluorescence into components with polarizations parallel and perpendicular to the polarization of the incident beam. The emission spectrum is dispersed by an imaging spectrograph, and detected with a resistive-anode imaging photomultiplier operated in a single photon counting mode. The time of arrival of a photon is derived from signals originating in the micro-channel plates that function as the "dynodes" of the photomultiplier, while the location of the centroid of the electron cascade on the anode of the detector indicates both the wavelength and polarization of the detected photon. Simultaneous acquisition of the time-resolved emission spectra for both polarization components is more efficient than conventional approaches and reduces the complications in data analysis that can arise when the properties of a sample change during the time when sequential data-sets are collected.

Keywords: emission spectra, fluorescence lifetimes, fluorescence polarization, fluorescence anisotrophy, synchrotron radiation

1. INTRODUCTION

Fluorescence spectroscopy is a powerful and sensitive tool for probing the properties of chemical and biological systems. Compared to other analytical methods, fluorescence offers a variety of experimental parameters: excitation and emission spectra, temporal profiles and polarization. As initially developed, these parameters are recorded sequentially, hence making complete characterization of the emission of a sample a laborious process. Recently, multielement detectors, *e. g.*, photodiode arrays and CCDs, have made possible the simultaneous recording of entire fluorescence spectra, but cannot respond to the temporal properties of fluorescence with picosecond resolution. Information about the local environment of the chromophore, including pH and dielectric constant, can often be inferred from

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fluorescence measurements. While static, or steady-state, measurements provide information regarding the time-integrated properties of the electronically excited state of the chromophore, time-resolved fluorescence spectroscopy, employing either time-correlated single-photon counting[1] or phase modulation[2] techniques, allows the dynamics of the electronically excited molecule to be probed. Thus, the capability of recording time-resolved fluorescence spectra provides a powerful tool for probing time-dependent structural and environmental physical properties. Furthermore, upon excitation with linearly polarized light, only electronic transitions having the proper transition dipole moment with respect to the direction of the excitation light are allowed. Resolution of the linearly polarized emission into components which are perpendicular and parallel to the direction of the excitation source allows the transition moment dipole of the emitting electronically excited state to be deduced. A measurement of this polarized emission as a function of emission wavelength and post-excitation pulse time yields an enormous quantity of information about the nature of the excited states involved, as well as the rotational dynamics of the chromophore.[2] Such measurements are valuable in studies of biological macromolecules, where information regarding the rotational times of the macromolecule, as well as the nature of chromophore/macromolecule interaction, can be deduced.

Until recently, recording the time-resolved fluorescence spectrum required recording the fluorescence decay at each individual emission wavelength of interest. Assembly of the entire time-resolved spectrum necessitated repeating this procedure at the individual wavelengths. Such measurements require a great deal of time and often result in lost spectral information. Furthermore, with fragile biological samples, possible photodegradation as a result of extended irradiation times raises concerns of sample integrity during the measurement process. Implementation of a resistive-anode single-photon-counting (RASPC) photomultiplier to simultaneously resolve the spectral and temporal properties of ultraviolet and visible fluorescence has been reported.[3-5] In this paper, we extend this technology to include the polarization of fluorescence. Our approach makes use of the two dimensional detection capabilities of a resistive-anode single-photon-counting photomultiplier, coupled with a spectrograph to spatially disperse the fluorescence spectrum along one direction and a Wollaston polarizer to separate the polarization components in the orthogonal direction on the photocathode of the detector. This implementation allows all of the information necessary to determine the time-resolved anisotropy spectrum of a fluorescent sample to be recorded simultaneously, thus significantly reducing data collection time. In addition, our approach permits the complete time-wavelength-polarization set to be viewed continuously during data collection.

2. EXPERIMENTAL

Anthracene (J. T. Baker, Inc., Phillipsburg, NJ) was recrystallized from ethanol prior to use. Ethanol was obtained from commercial sources and its purity checked by the measured the UV cut-off of the blank solvent. Ludox was from DuPont Chemicals (Wilmington, DE).

Our instrument for simultaneously recording the spectral and temporal properties of a detected fluorescent photon has been described previously.[5] Briefly, the system utilizes a position-sensitive detector, comprised of five microchannel plates in series, with a resistive anode for detection of the photoelectrons. The sample fluorescence is dispersed and focused along one dimension of a bialkali photocathode of an ITT F4146M detector (Quantar Technology, Inc. Santa Cruz, CA) using a Model 250 Imaging Spectrograph (Chromex, Inc., Albuquerque, NM). A timing pulse, induced from the photoelectron cascade, is generated across a voltage divider circuit connected to microchannel plate.

After proper amplification (x 100) and shaping with an ORTEC Model 474 Timing Filter Amplifier (EG&G ORTEC, Oak Ridge, TN), this timing pulse starts the voltage ramp in a time-to-amplitude converter (TAC) (EG&G ORTEC Model 467). The voltage ramp is stopped by pulses generated at a frequency equal to 1/9 that of the radio frequency power supply that maintains the energy and grouping of the 9 bunches of electrons circulating in our synchrotron storage ring. Thus, one stop pulse is received for each set of nine excitation pulses. The analog signal corresponding to the (stop—start) time difference is digitized by an external analog-to-digital converter (ADC). Concomitantly, the x and y coordinates of the detected photon are determined by a position analyzer from the currents at the four corners of the resistive anode. An analog signal, corresponding to the x position, where x is the dimension in which the fluorescence spectrum is dispersed by the Chromex spectrograph, is digitized by a second ADC. The spectral and timing information are stored in the two-dimensional histogramming memory of a multiparameter analyzer (MPA) (Quantar Technology).

We have modified this system to add the capabilities of resolving the polarization properties of the sample fluorescence. A schematic of the spectrometer is shown in Figure 1. Monochromatic light from the U9B, quartz-windowed, bending magnet port at the National Synchrotron Light Source is selected with a SPEX Model 1680 0.227 m double monochromator, equipped with holographic gratings blazed at 300 nm (SPEX Industries, Edison, N. J.). The light is linearly polarized by a Glan-Thompson polarizer and focused onto the sample position. The sample can be contained in a quartz cuvette, a quartz capillary mounted in a cryostat, or can be a free standing solid. The vertically and horizontally polarized components of fluorescence (plus phosphorescence and scattered light) from the sample are spatially separated in the vertical (y) dimension using a Wollaston polarizer with 10° deviation (Spindler & Hoyer, Milford, MA). The two images, corresponding to the parallel and perpendicular components of the fluorescence, are focused onto the entrance slit of the spectrograph. The position was adjusted such that the images were symmetrically spaced about the middle of the vertical slit.

The wavelengths of fluorescence, for each polarization, are dispersed across the horizontal (x) dimension of the position-sensitive detector. The active area of the detector is 1" ϕ. The maximum count rate of the RASPC PMT is $\approx$ 100 kHz, which is much less than the $\approx$ 50 MHz excitation pulse rate, so proper single-photon counting statistics are assured. Hence, the timing pick-off, described above, was utilized to resolve the temporal properties of the detected photon in a time-correlated single-photon counting implementation (see Figure 1). The analog signal (0 - 10 V) from the TAC was digitized (10 bit resolution) with a Canberra (Canberra Industries, Meriden, CT) 8075M 100 MHz Wilkinson ADC, operating in PHA mode. Simultaneously, the spectral and polarization properties of the detected photon are resolved using the position-sensitive capabilities of the detector. Analog signals (0 - 5 V), corresponding to the x and y coordinates of the photon, are digitized as shown in Figure 1. The temporal, spectral, and polarization properties of the detected photon were reduced to two independent inputs from three external ADC's as shown in Figure 1. The information about the vertical position of the detected photon with respect to the center of the spectrograph entrance slit is carried in the most significant bit (2^9 in Figure 1) of the "y" ADC. This bit replaces the most significant bit of the "x" ADC (Canberra 8075M operating in SVA mode). The resolution of the "x" ADC is adjusted such that the range of voltages representing the wavelength of the photon fall into the lower half of the input range. This insures that the most-significant bit of the "x" ADC output is always zero, and thus contains no meaningful information. The digitized spectral (9 bit resolution per polarization) and temporal information (10 bit resolution) were recorded in the 2D 1024 x 1024 histogramming memory of the multiparameter analyzer memory, operating in multi-dependent mode. The resultant data thus consists

of two time-resolved emission spectra in the multiparameter analyzer memory, corresponding to each of the polarization components. Wavelength calibration of the position-sensitive detector was accomplished by scattering monochromatic light, at a series of wavelengths, from the calibrated SPEX excitation monochromator using an aqueous suspension of ludox. The resultant linear plot of channel number vs. scattered photon wavelength, was used for the wavelength calibration.

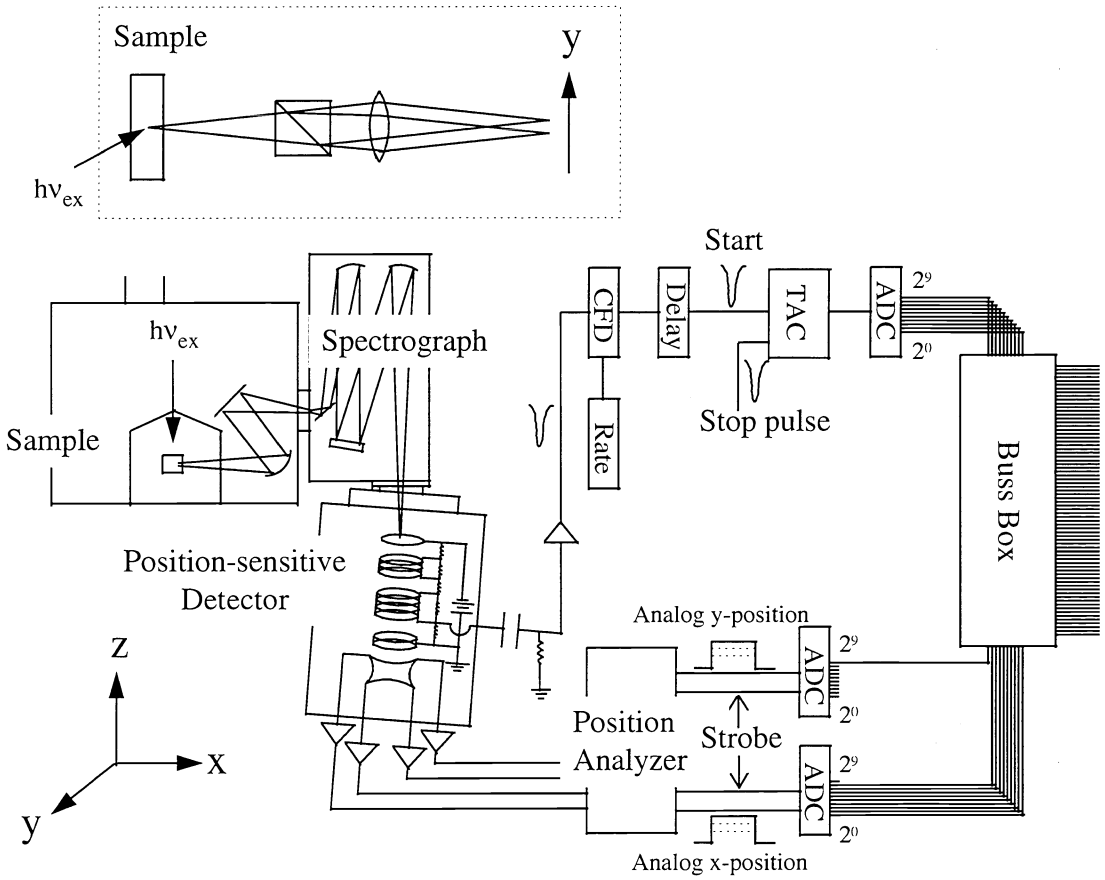

Figure 1 **Instrumental layout and schematic of the fluorescence spectrometer for time-resolved anisotropy measurements. Inset shows cross-section of the vertical (*y*) dimension in which the components of the luminescence which are parallel and perpendicular to the linearly polarized excitation light are spatially separated by a Wollaston polarizer and focused on the spectrograph entrance slit. For clarity, the mirrors actually used to form an image of the fluorescence is replaced by an equivalent lens in the insert.**

Instrument response functions for each polarization, as a function of time and wavelength, were obtained by scanning the excitation monochromator to zero-order and scattering the white light with a suspension of Ludox in water. In this manner, an instrument response function, wavelength-integrated over the same region as the fluorescence decay of interest, was obtained. Fits to an exponential decay model were obtained by iterative reconvolution of the measured instrument response function with a single or double exponential decay model.

Correction factors ($G = I_V/I_H$), accounting for different optical and detection paths of each polarization, were obtained as a function of wavelength by measuring the fluorescence anisotropy from

an isotropic solution of a fluorophore. Two fluorophores were employed, sodium salicylate and anthracene in ethanol at room temperature, for this purpose. The measured correction factor, G = 1.184, was found to be independent of detected wavelength and fluorophore employed.

Fluorescence from the sample, which was contained in a 1 cm x 1 cm quartz cuvette, was collected at right angles with respect to the excitation. A 1 mm Schott filter (WG -320) (Esco Products, Inc., Oak Ridge, NJ) was inserted between the sample and emission collection optics to minimize scattered excitation light. The sample temperature was controlled using a water-jacketed cuvette holder, with a Lauda K-2/R water circulating bath (Brinkmann Instruments, Inc., Wesbury, NY). At low temperatures, the sample chamber was purged with dry nitrogen to prevent condensation on the outside of the cuvette. Excitation bandwidths varied between 2 and 3 nm in these measurements.

The MPA is built on a ISA bus computer interface adapter and mounted in a computer with an 80386 microprocessor (Intel, Santa Clara, CA) using the MS-DOS operating system (Microsoft Corp., Redmond, WA). The ≈ 2 Mbytes data-sets containing time, wavelength and polarization data were stored temporally on the hard disk of the data acquisition prior to archival storage on a SPARCserver 10 (Sun Microsystems, Mountain View, CA). Data were transferred from the data acquisition computer to the SPARCserver, and from the SPARCserver to one of a number of data analysis workstations *via* a computer network. Our system of networked computers for data acquisition, storage, and analysis has been described elsewhere.[6] Two-dimensional fluorescence data sets were analyzed with the aid of the program Origin (Microcal Software, Inc., Northampton, MA).

3. RESULTS

3.1. Spectrometer Description.

Shown in Figure 1 is an instrumental layout of the fluorescence spectrometer for time-resolved anisotropy measurements. The ***x*** and ***y*** coordinate of the detected fluorescent photon, determined by the position-sensitive detector and analyzer, record the photon wavelength and polarization as described above. Simultaneously, a timing pulse, coincident with the incident photon, is derived from the electron-cascade in the detector. With proper optical alignment to center the two images corresponding to the parallel and perpendicular components of the fluorescence about the middle of the vertical entrance slit of the monochromator, the data are reduced to two independent inputs into the multi-parameter analyzer. Photon counts recorded in the top and bottom (***y*** in Figure 1) half of the detector correspond to mutually perpendicular linearly polarized components of the emission. The spectral information of these photons is given by the ***x*** coordinate of the position-sensitive analyzer system.

3.2. *x-y* image of detected photons.

Shown in Figure 2 are the ***x*** and ***y*** mapping of the photon counts scattered from an aqueous suspension of ludox. The data were collected with the excitation polarizer oriented in the vertical direction, *i. e.*, perpendicular to the plane defined by the principal axes of the excitation and emission beams. (For the light from port U9B, this corresponds to the preferred polarization for the synchrotron radiation). For the data shown in Figure 2, scattered light from four separate excitation wavelengths were recorded sequentially. The bandwidth of the excitation source was 0.6 nm for the scattered light.

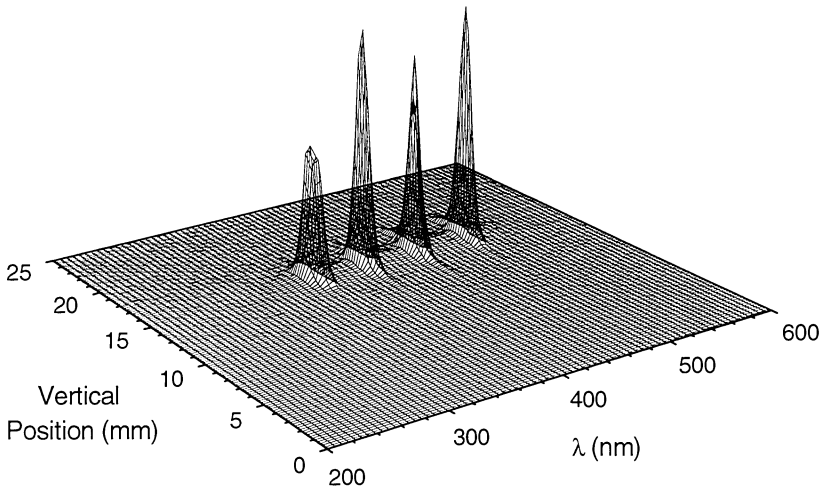

Figure 2 **Three-dimensional representation of the wavelength (x) and vertical position (y, channel) of synchrotron radiation which was monochromated and scattered from a Ludox suspension. Excitation wavelengths of 325, 375, 425, and 475 nm were employed for the data shown in the figure. The excitation polarizer was oriented vertically, so only vertically polarized photons were scattered, and the Wollaston polarizer routed all vertically polarized photons to the upper half of the detector's photocathode. Thus, all counts appear at y values greater than the center value.**

The resolution of the detected monochromatic light was ca. 5 nm ($\Delta\lambda$ at the FWHM of the recorded peak) and, thus, limited by the spectrograph/detector optical configuration. With the excitation polarizer oriented vertically, counts are only detected in the top half of the detector. The result verifies the proper operation of the scheme shown in Figure 1, as well as the optical alignment of the polarizers.

In room temperature fluid solution, most chromophores which are not associated with a macromolecule freely and rapidly rotate faster than the lifetime of the emitting state. Thus, upon electronic "photoselection" with linearly polarized light of molecules with the proper transition moment dipole, the molecules will rapidly reorient and emit a random (isotropic) distribution of linearly polarized photons. In the absence of any rotation, the anisotropy (r), defined by Equation 1, reduces to the theoretical limiting maximum of 0.4.[2] For a completely isotropic distribution of fluorophores, the intensities of linearly polarized light which is parallel and perpendicular to the excitation source are equal. The G term in Equation 1 accounts for different detection efficiencies due to the different optical paths that the two linearly polarized components transverse.

$$r = (I_V - GI_H)/(I_V + 2GI_H \qquad \text{Equation 1}$$

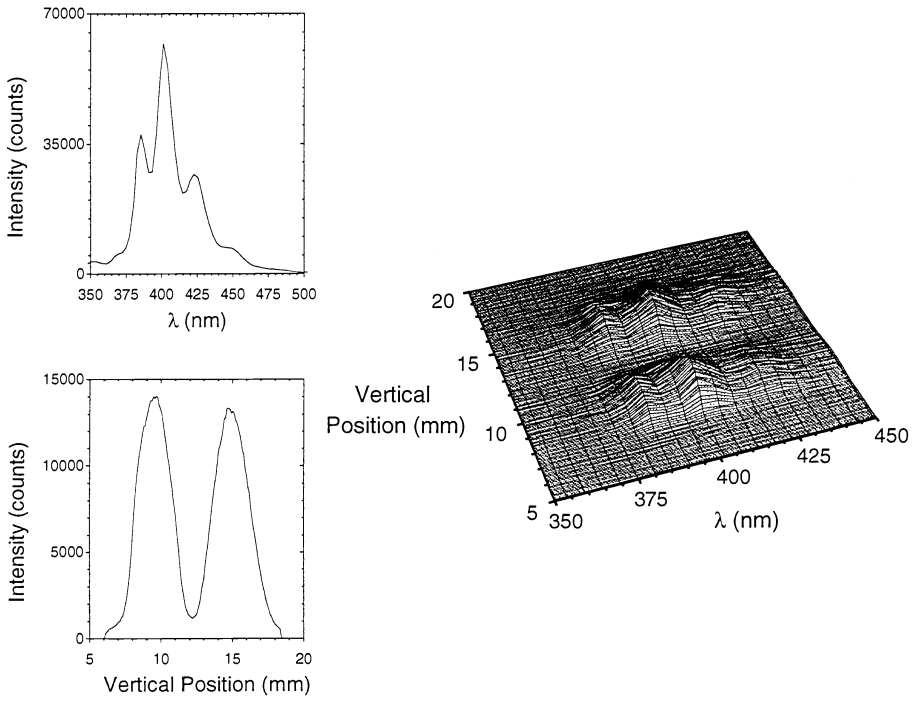

Figure 3 **Three-dimensional representation of the wavelength (*x*) and vertical position (*y*, channel) of fluorescence from anthracene (*ca.* 10 μM) in ethanol. 290 nm synchrotron excitation was employed, with the excitation polarizer oriented vertically.**

The ***x*-*y*** image of the isotropic fluorescence from a solution of anthracene in ethanol at room temperature is shown in Figure 3. Two symmetrically distributed spots are produced in the vertical (***y***) dimension, corresponding to the vertically and horizontally polarized components of fluorescence. Furthermore, the characteristic vibrational structure of this fluorophore is clearly resolved. Shown in the Figure are two virtually equal bands (intensity vs. vertical position) from the isotropic solution in the wavelength-integrated cross-section. The relative intensities of the two bands (I_V/I_H = 1.184) was close to unity, as expected. Any deviation from unity was taken as a measure of the different detection efficiencies of the two components and used as the correction factor (***G***) in Equation 1.

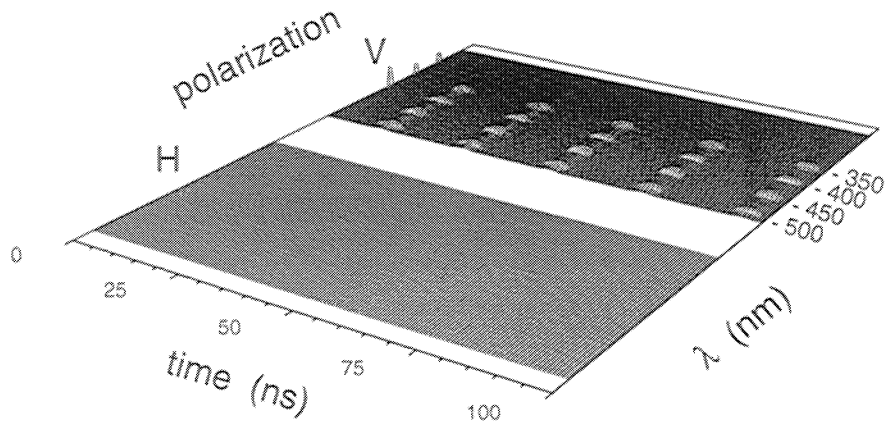

Figure 4 **Three-dimensional representation of the wavelength (x) and arrival time (with respect to the excitation pulse) of synchrotron bunches (5 shown) scattered from a Ludox suspension. Excitation wavelengths of 350, 400, 450, and 500 nm were employed with the excitation polarizer vertical**

3.3. Time-resolved polarized fluorescence measurements.

3.3.1. Scattered excitation

Shown in Figure 4 is the intensity of monochromatic scattered light from a suspension of Ludox in water, as a function of time and wavelength. Scatter from five synchrotron bunches, at the four indicated wavelengths, is shown. Data were collected as above by successively scanning the excitation monochromator to the desired wavelength. The figure shows the time-resolved spectra, resolved into components which are parallel and perpendicular to the direction of the linearly polarized excitation light. As the excitation polarizer is set to pass vertically polarized light, counts are recorded only in the

upper area of the detector plane, as scattering from a dilute solution does not modify the plane of polarization.

3.3.2. Anthracene fluorescence

Shown in Figure 5 is the polarized, time-resolved emission spectrum of an isotropic solution of anthracene in ethanol at room temperature. As expected, the components of the fluorescence from an isotropic fluorophore which are parallel and perpendicular to the direction of the linearly polarized excitation light are of equal intensity and serve as a check of our system. The fluorescence intensity as a function of time, wavelength, and polarization shown in Figure 5, is independent of the direction of the polarization of the excitation employed.

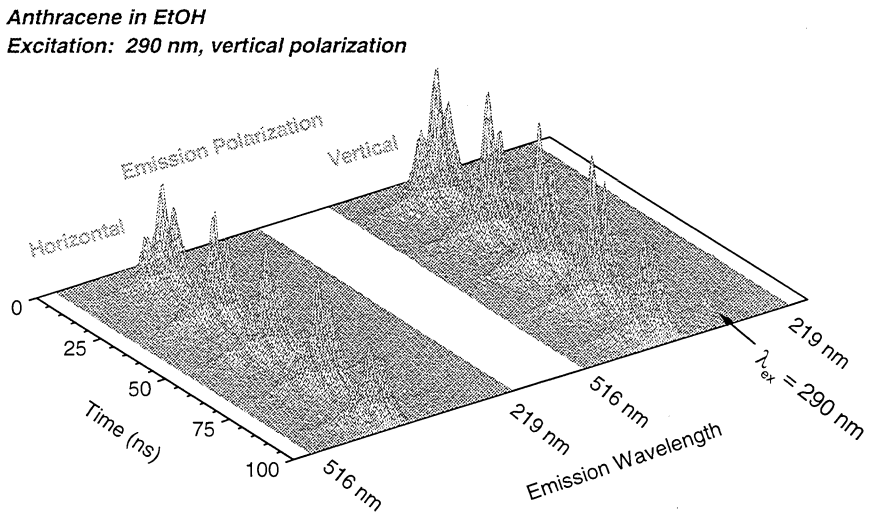

Figure 5 **Three-dimensional representation of the wavelength (x) and arrival time (with respect to the excitation pulse) of the fluorescence of anthracene in ethanol from 5 synchrotron excitation bunches. 290 nm excitation was employed with the excitation polarizer oriented vertically.**

4. CONCLUSIONS

We have demonstrated the use of a resistive-anode single-photon-counting photomultiplier to simultaneously record the spectral, temporal, and polarization properties of fluorescence spectra. This implementation reduces data collection times for recording time-resolved anisotropy spectra, eliminating

the need for scanning of an emission monochromator and independent or dual-channel measurements of fluorescence polarization. The high brightness of the synchrotron source and reduced data acquisition time offer numerous advantages for measuring rotational depolarization times in biological samples, where sample volumes are often small, and the risk of photodegradation is a concern.

5. ACKNOWLEDGMENTS

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