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Microprocessor-controlled photon-counting spectrofluorometer

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The construction details and performance characteristics for a fluorescence spectrophotometer are described. The system utilizes proton counting for signal detection and an Apple II microcomputer for data acquisition and analysis. Data-acquisition techniques are given along with typical excitation, emission, and polarization spectra. Particular reference is made to application of the instrument to fluorescence studies of protein/polymer films.

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INTRODUCTION

Fluorescence measurements are very useful for the study of chemical and biological systems because of the high sensitivity of the fluorescence emission to both static and dynamic properties of the system.[1] However, the weak intensity of the fluorescence given off by biologically interesting samples often requires very sensitive signal detection. Furthermore, one would like to acquire the fluorescence data in a way that makes it easily available for a variety of analyses. Photon counting techniques have been developed that achieve the required sensitivity to the weak signal.[2,3] Instruments have also been described which utilize low-cost small computers for simple yet powerful control of the acquisition of fluorescence spectra and for analysis of the data.[4,5] We describe here an instrument built in our laboratory that utilizes a commercially available optical system and our own photon-counting detection, data-acquisition, and wavelength control electronics. We interfaced these with an Apple II microcomputer (Apple Computer, Inc., Cupertino, California). The result is extensive flexibility and ease in the acquisition, storage, display, and analysis of fluorescence measurements since all these operations can be performed through microcomputer software and peripherals. We are now using the instrument for studies of the internal dynamics of proteins imbedded in polymer films.

I. OPTICS AND SIGNAL DETECTION

Figure 1 shows the overall layout of the spectrofluorometer. An optical module designed for fluorescence measurements (Model OP-450) and two companion holographic grating monochromators (Model MC-320) were purchased from SLM Instruments (Urbana, Illinois). The monochromators have a wavelength range from 200 to 900 nm. They were calibrated using a He–Ne laser (6328 Å). Their external wavelength dials remained calibrated to 0.5 nm over a year of frequent use. The SLM optical module contains shutters, lenses, and rotatable Glan–Thompson polarizers in the excitation pathway and in two emission pathways oriented at 90° to the excitation.

An optical pathway for a lamp reference signal allows monitoring of the lamp intensity (see Fig. 1). The design of this module makes it possible to take emission and excitation spectra and both L- and T-format polarization measurements. The lamp presently used is a 150-W water-cooled Xenon arc (Photochemical Research Associates, London, Ontario). For UV work requiring greater intensity, an air-cooled 200-W Mercury arc lamp is available for use (powered by Opti-Quip Model 1022) yielding about three times the intensity of the PRA lamp in the 280 nm region. The excitation and emission holographic grating monochromators greatly reduce stray light, but because this is critical for the protein/polymer film measurements, additional stray light filtering is needed. Presently, a Jarrell–Ash model 82-410 monochromator set to wide bandwidth is inserted as a bandpass filter between the lamp and the excitation monochromator.

The fluorescence emission and lamp reference signals are detected in the UV and visible by Amperex (Philips)
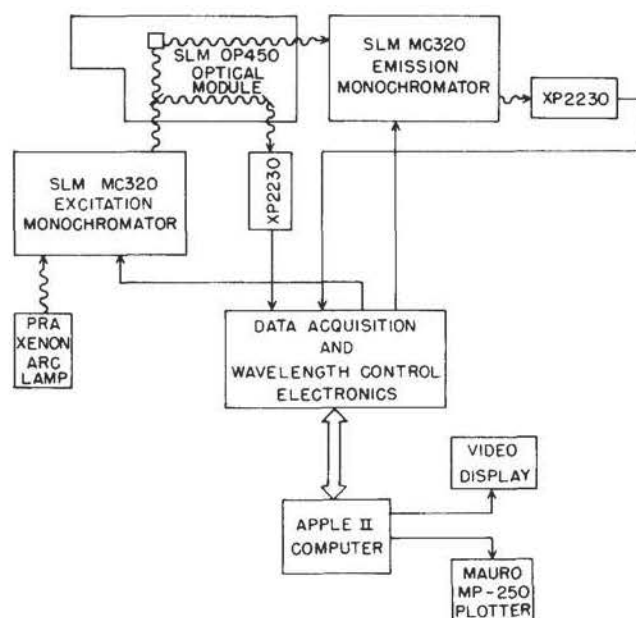

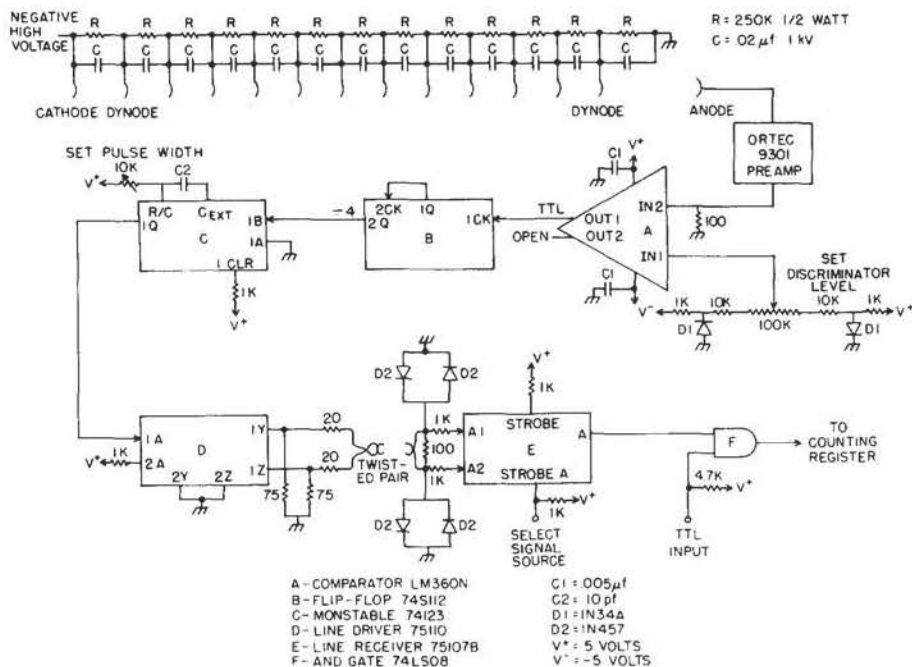FIG. 1. Overall layout of the spectrofluorometer.

FIG. 2. The reference signal detection circuit, with the photomultiplier dynode chain at the input. Grounding strobe A on the 75107B selects TTL input by setting output A to a high level. The output of this circuit enters the input of the circuit in Fig. 3. The emission PMT circuit is identical to and completely separate from the circuit shown here, except it uses the other half of the same 75107B dual line receiver.

A — COMPARATOR LM360N
B — FLIP-FLOP 74S112
C — MONSTABLE 74123
D — LINE DRIVER 75110
E — LINE RECEIVER 75107B
F — AND GATE 74LS08

C1 = .005 μf
C2 = 10 pf
D1 = IN34A
D2 = IN457
V⁺ = 5 VOLTS
V⁻ = −5 VOLTS

XP2230 photomultiplier tubes (PMT) wired in a linear dynode chain. This PMT was selected for its low dark count and high sensitivity. Hamamatsu R928 photomultiplier tubes are available for use where extended red spectral response is desired. The XP2230 PMT is operated near 2000 V by a regulated high-voltage supply (Northeast Scientific model RE-3010 or Fluke Model 405B). Output current pulses corresponding to single photons are amplified ten times by an Ortec model 9301 fast preamplifier and sent to a level comparator LM360 located in the PMT housing (Fig. 2). The comparator reference level is set by a trimpot (with diode temperature compensation) so that the measurement of dark photoelectrons is minimized and the fluorescence signal is maximized. The TTL output of the comparator is divided by four using a dual Schottky flip-flop 74S112 whose output triggers a monostable 74123. The pulse generated by the monostable is sent by a line driver 75110 along a twisted pair cable to a line receiver 75107B. The line driver/receiver combination is used to minimize stray noise and degradation in the detected signal during passage from the PMT housing to the signal-counting electronics. Diodes (IN459) and 1 k and 20 Ω resistors at the ends of the twisted pair cable prevent large current surges (such as those induced by a 25-kV lamp igniter) from damaging the line receiver.

The frequency response of the circuit of Fig. 2 has been measured at its output. Using as input 20-ns-wide 20-mV peak pulses generated by a Wavetek Model 166 50-MHz pulser, a maximum allowable input frequency of 22 MHz was recorded. With phototubes as input pulse sources, counting rates have been observed at the output of as much as 900 KHz before signal saturation occurs. From statistical considerations, linearity in the PMT response is expected to be within 1% up to a counting rate of 250 KHz.[6] Linearity was experimentally observed up to about 300 KHz. Since most of the experiments with this instrument are at very low light levels, a few KHz at most,

the dark count rate of the XP2230 of about 25 Hz at room temperature is a more significant contribution to the signal than pulse pileup or any other nonlinearities in the PMT response. The dark count is compensated for by subtracting it from the fluorescence signal using the microcomputer.

II. DATA ACQUISITION

The data acquisition and wavelength control electronics were built in a unit separate from the microcomputer, and communicate with the computer through the computer interface (Fig. 3). The wavelengths of both SLM monochromators are visually monitored through counting circuitry by means of encoders attached to the monochromator grating stepper motors. Limit switch circuitry prevents driving the gratings beyond their physical limits of motion. The stepper motor driving circuit was one provided by the department electronics shop.

Data acquisition is done in a ratiometric mode. The emission and reference PMT signals are sent to their respective signal counters (Fig. 3). Data acquisition is computer initiated by a reset of these counters. With the ref-
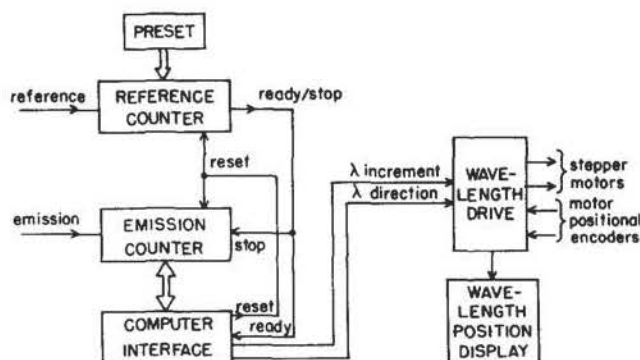

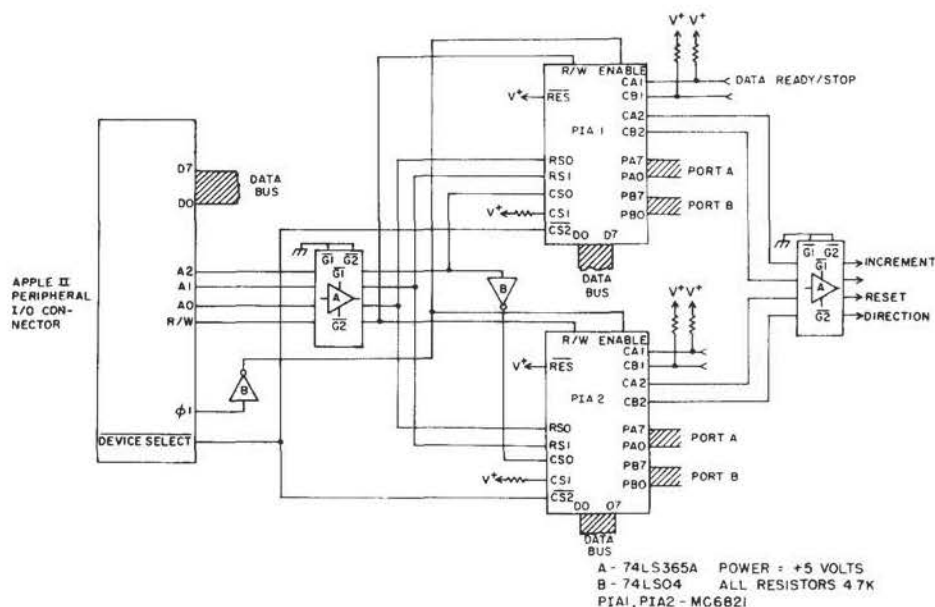FIG. 3. Outline of the data-acquisition and wavelength drive process.

FIG. 4. Computer interface to the Apple II microcomputer.

A - 74LS365A   POWER = +5 VOLTS
B - 74LS04   ALL RESISTORS 4.7K
PIAI, PIA2 - MC6821

erence counter initialized to count down from a preset value, signal accumulates in both counters until the preset reference count is acquired. The contents of the emission counter is then transferred through the computer interface to the microprocessor. Although the computer receives only one number, the measurement is effectively the ratio emission/reference with the count value of the reference set to a constant. This ratiometric approach compensates for lamp intensity variations when measuring fluorescence emission spectra and keeps the statistical noise of the reference signal constant. For a sufficiently large reference count, the noise of the reference is negligible, and the noise of the data acquired becomes just the square root of the value of the emission count. A TTL input line to the reference counter (see Fig. 2) provides a constant integration time option for cases where this is preferred over using the exciting light as the reference.

In a wavelength scan, the computer acquires a count value at a particular wavelength as described above and then increments the excitation or emission monochromator wavelength position through the stepper motor driving electronics. The wavelength can be incremented in either direction in steps of 0.25 nm. When the increment is completed, count acquisition is again initiated. Although the emission count value for a single point is limited to 16 bits (65535 counts) due to the chosen size of the emission counter, repetitive scanning of the wavelength range of a spectrum increases the dynamic range of counting up to the essentially infinite limit allowed by the microcomputer memory and software. Single wavelength measurements can be done with the same dynamic range by simply deleting the wavelength increment instruction from the computer program.

III. COMPUTER INTERFACE AND SOFTWARE

The computer interface is designed specifically for the Apple II microcomputer utilizing the Motorola 6502 microprocessor and memory-mapped I/O (Fig. 4). The interface resides in one of the computer's peripheral I/O slots. Two 6821 Peripheral Interface Adapters (PIA) and a few logic and buffer gates provide two flexible 16-bit parallel I/O ports ("A" and "B") with associated control lines. Communication with the computer interface is done primarily through machine language routines. Only one 16-bit port is presently used for the data input. The unused port allows for future expansion of the computer's communication with the counting electronics. Such expansion is envisioned to include a third PMT for T-format polarization measured against a time reference.

Our present computer system includes the 48K Apple II with Language Card, two 5¼ in. Disk II floppy disk drives, a B/W video monitor, and an MP-250 Proac digital plotter (Mauro Engineering, Mt. Shasta, California). Programming is done in BASIC and PASCAL. Presently written software does real-time display of spectra on the video monitor and provides a flashing cursor on the screen during signal counting so that exact peak locations and intensities can be found while the data are being acquired. Figure 5 shows a typical screen display. As many as 80 spectra can be stored on a single floppy disk, and are easily recalled for display or further analysis. The choice
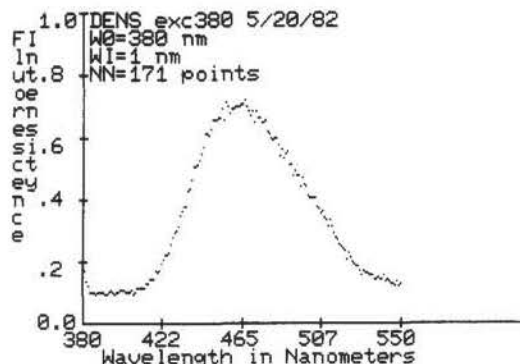

FIG. 5. Typical screen display of spectra on the video monitor. The cursor is not shown. An Epson MX-80 printer was used to make this hard copy of the Apple graphics.

of arithmetic operations that can be performed on spectra is limited only by computer speed and memory, both of which have been quite adequate for all the calculations yet envisaged. For permanent output, the digital plotter produces high-quality graphs (see Figs. 6–10).

IV. EMISSION SPECTRA

An emission spectrum of the protein Azurin in 1-mM phosphate buffer is shown in Fig. 6. The raw data (discrete points) are shown for both Azurin in buffer and the buffer alone. The smooth curve is the result of first subtracting the buffer level from the Azurin spectrum and then applying a digital filter which reduces the noise from statistical scatter in the data.[7,8]

Emission spectra must be corrected for the instrumental wavelength response of the emission optics, monochromator, and PMT. To do this correction a quartz–halogen tungsten filament lamp was used (Model 254A Serial No. L-209, Optronics Laboratories, Inc., Silver Springs, Maryland).[9] The lamp has a known spectral irradiance in the wavelength region from 250 to 750 nm which was measured by detectors calibrated at the National Bureau of Standards. The lamp was positioned in the unused emission arm of the optical module with all lenses and the polarizer removed from this arm. Its output was scanned across the wavelength region using the data-acquisition method previously described, with the reference counter driven by a TTL signal generator to provide a constant integration time across the scan. The instrumental response was found at each wavelength by dividing the measured intensity of the lamp at that wavelength by the lamp flux calculated from the known spectral irradiance for that wavelength. Similarly then a corrected spectrum is found by dividing the measured spectrum by the instrumental response. The instrumental response is
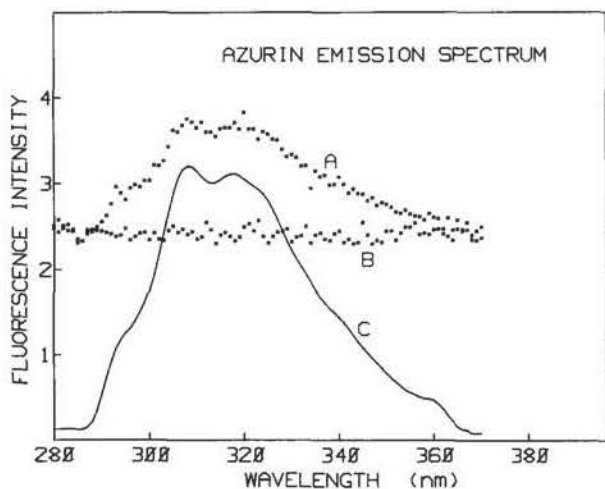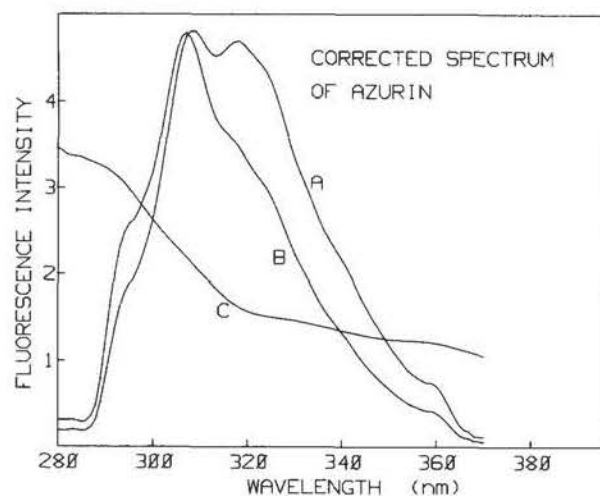

FIG. 7. Azurin spectrum: same sample as Fig. 6. (A) Data as observed, after background subtraction and filtering (same as curve C in Fig. 6). (B) Spectrum corrected for instrumental response. (C) Multiplicative instrumental correction curve (reciprocal of that described in the text).

not the same for polarized and unpolarized emission so different response curves must be computed for each choice of polarization.

In our instrument the response curve is primarily determined by the spectral sensitivity curve of the XP2230 photomultiplier tube, and peaks at approximately 400 nm. Uncorrected and corrected spectra of an Azurin solution and of a Perkin–Elmer Rhodamine B calibration block[10] show the effect of the instrumental correction at the ultraviolet and at the red regions, respectively, of the instrument's sensitivity (Figs. 7 and 8). It is in these regions that we expect the largest errors to occur in corrected spectra. The corrected Azurin peak at 307 nm and shoulder at 295 nm are similar to published values of 308 and 298 nm.[11,12] The corrected Rhodamine B peak at 571 nm corresponds closely to the Perkin–Elmer value of 573 nm.[10] We did not attempt to duplicate the exact experimental conditions of the published values in either case.

FIG. 6. Emission spectrum of Azurin: from Pseudomonas aeruginosa, 0.03 mM in 1 mM pH 7.0 phosphate buffer. λ_{exc} = 280 nm, excitation bandwidth 8 nm, emission bandwidth 2 nm. (A) Data as acquired (1400 counts full scale), (B) typical buffer spectrum, (C) fluorescence after subtracting the buffer and applying a digital filter to reduce noise (560 counts full scale). Since the buffer spectrum here is flat, its average value was subtracted from each point of the Azurin spectrum rather than doing a point-by-point subtraction of the two spectra.
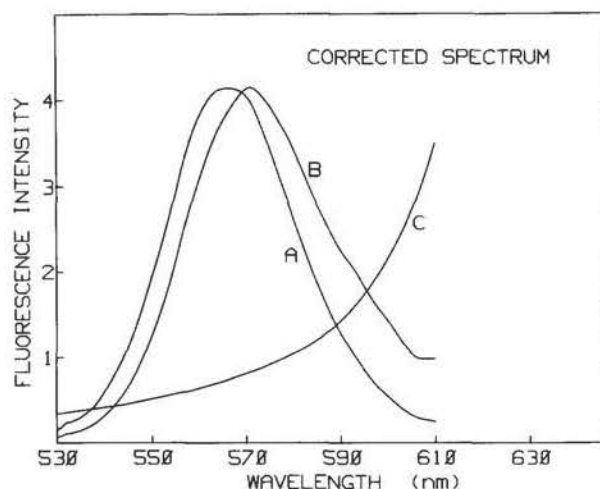

FIG. 8. Perkin–Elmer calibration block: Rhodamine B imbedded in polymethylmethacrylate (PMMA), 0.003 mM. λ_{exc} = 500 nm, excitation bandwidth 8 nm, emission bandwidth 2 nm. (A) Uncorrected and (B) corrected spectrum. (C) Multiplicative instrumental correction curve.

For example, a change in our conditions for the Rhodamine B block gave us a second corrected curve peaking at 575 nm. The variations in these regions are of no consequence to us for the studies we presently plan to do, and they can be easily improved through the use of the R928 PMT which has a broader spectral sensitivity.

V. EXCITATION SPECTRA

Several measurements are needed to acquire a corrected excitation spectrum. The ratiometric mode is used as described in the data-acquisition section. First the fluorescence emission of a concentrated Rhodamine B solution (quantum yield = 1) is measured as a function of the excitation wavelength in the region of interest. A measurement of the emission PMT dark count over the same region must then be subtracted from this. The result (A) is the product $L(\lambda) \times P(\lambda)$, where $L(\lambda)$ is the wavelength dependence of the lamp output and $P(\lambda)$ is the wavelength dependence of the reference photomultiplier tube. Then a background scan (B) is made (using, for example, buffer solution, or film without fluorophore) over the same excitation region with the emission monochromator wavelength set in the fluorophore emission band. This measures the product $B(\lambda) \times L(\lambda) \times P(\lambda)$, where $B(\lambda)$ includes buffer emission and emission PMT dark count. Finally the fluorophore is scanned (C), yielding the product $F(\lambda) \times L(\lambda) \times P(\lambda)$, where $F(\lambda)$ is the fluorophore emission plus buffer and/or dark count contributions. The computer calculation $(C - B)/A = F(\lambda) - B(\lambda)$ gives the corrected excitation spectrum. This will be accurate if the excitation source is stable in intensity over the period of one spectrum and stable in wavelength response over the period of a complete measurement. These requirements are satisfied with the lamp in our system. The excitation spectrum of Azurin is shown in Fig. 9, and as in the emission case corresponds to that in the literature.[11,12]

VI. POLARIZATION MEASUREMENTS

The L format was chosen for film polarization measurements. This is the same optical configuration as Fig. 1, in contrast with the so-called "T-format", where the emission is viewed simultaneously through both emission arms of the SLM optical module. The L format is advantageous for film polarization measurements because it allows the emission monochromator to be used for filtering out scattered light. The L format also minimizes possible artifacts arising from the emission passing through the quartz slide on which the film is deposited, as it would have to do in order to be observed in the other arm of a T-format measurement. Note the instrument is not limited to L format. T format can be done by simply moving the reference PMT to the empty emission arm and acquiring data by using the same data-acquisition procedure.

The L-format polarization measurement requires four orientations of the polarizers. The intensities $I_\parallel$ and $I_\perp$ of the emission are measured, with the excitation polarizer
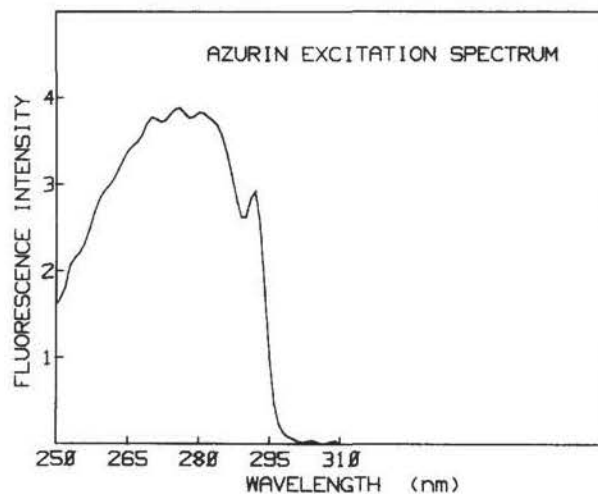

FIG. 9. Excitation spectrum of Azurin (same sample as Fig. 6). λ_{em} = 320 nm, excitation bandwidth 1 nm, emission bandwidth 8 nm.

oriented first parallel (V) then perpendicular (H) to the laboratory axis. (V = vertical, H = horizontal.) In principle, when the excitation polarizer is in the H position, the two emission measurements are identical, that is, $(I_\parallel)_H = (I_\perp)_H$. This is because the electric vector of the exciting light is in the direction orthogonal to the electric vector transmission direction of the emission polarizer. In practice, the optical response of the emission arm differs for parallel and perpendicular light, so a normalization factor corrects for this in the following manner[3]:

$$\frac{\left(\dfrac{I_\parallel}{I_\perp}\right)_V}{\left(\dfrac{I_\parallel}{I_\perp}\right)_H} = \left(\frac{I_\parallel}{I_\perp}\right)_{Norm},$$

$$p = \frac{\left(\dfrac{I_\parallel}{I_\perp}\right)_{Norm} - 1}{\left(\dfrac{I_\parallel}{I_\perp}\right)_{Norm} + 1}.$$

Polarization data are acquired in the nonwavelength-scanning mode. The computer corrects each of the four measurements above for background counts before calculating the polarization. The polarizers were aligned using a glycogen scattering solution. The average polarization of the scattering at 500 nm was 0.993. Low polarization was checked using 2-Diethylamino-5-napthalenesulfonate (DENS) in pH 7 phosphate buffer, which gave a measured polarization of -0.002 ± 0.005 when excited at 380 nm. These values are in agreement with other reported calibrations.[3]

VII. FILM STUDIES

We are interested in the effects certain environmental changes will have on the internal dynamics of proteins. The desired environmental control and fluorescence measurements can both be done on proteins if they are imbedded in a thin (tens of microns in height) polymer matrix.
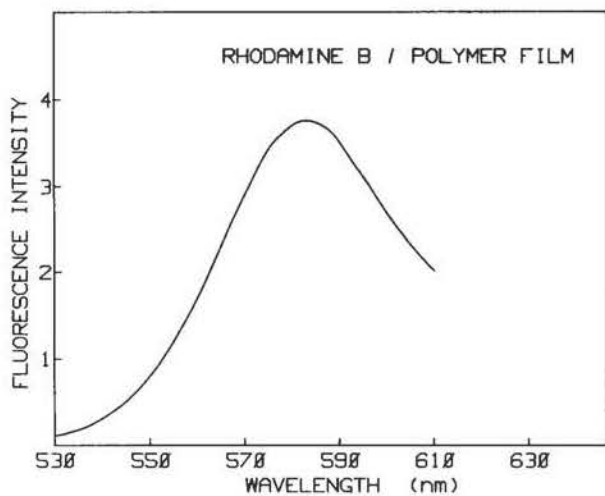
FIG. 10. Corrected emission spectrum of Rhodamine B imbedded in poly (vinyl methyl ether/maleic anhydride) 1:1 copolymer. Concentration 0.5 mM in dry film. λ_{exc} = 500 nm, excitation bandwidth 8 nm, emission bandwidth 16 nm.

The polymer we use is poly (vinyl methyl ether/maleic anhydride) 1:1 copolymer, a gift from M. Goodman (UCSD). Because of the complexity of emission and polarization measurements in such films preliminary studies have been done using simple fluorophores such as Rhodomine B instead of proteins. A corrected emission spectrum of a Rhodamine B/polymer film has a peak at 583 nm (Fig. 10). The polymer film without fluorophore excited at this same wavelength shows no fluorescence. The shift of the Rhodamine B/polymer emission peak to a longer wavelength relative to the Rhodamine/PMMA block emission peak (see Fig. 8) is likely due to the different effects that the polymer and PMMA environments have on the fluorophore. Excitation polarization mea-

surements on the two samples yield similar data, indicating that the higher concentration of Rhodamine in the film is not resulting in fluorophore–fluorophore interaction. The polarization measurements furthermore indicate that a fluorophore in the polymer film cannot rotate on the nanosecond time scale of the fluorescence, and indicate that the polymer film itself does not depolarize light. These facts will be useful in interpreting the more complex motions expected for proteins in the polymer films.

ACKNOWLEDGMENTS

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