A PHASE-SHIFT FLUOROMETER USING A LASER AND A TRANSVERSE ELECTROOPTIC MODULATOR FOR SUBNANOSECOND LIFETIME MEASUREMENTS

I. SALMEEN AND L. RIMAI, *Scientific Research Laboratory, Ford Motor Company, Dearborn, Michigan 48121 U.S.A.*

ABSTRACT We describe a simple phase-shift fluorometer using continuous laser excitation. The laser enables the use of a transverse mode electrooptic modulator with a half-wave retardation voltage of about 200 V (in contrast to many kilovolts of longitudinal modulators) at frequencies up to 100 MHz. The modulated fluorescence signal is detected, after passing through a double monochromator, by a photomultiplier tube feeding a radio frequency (RF) tuned amplifier. The RF phase is then determined by phase-sensitive detection using a double balanced mixer with the reference obtained from a PIN photodiode-tuned amplifier combination which detects light split off from the main exciting beam. The laser and double monochromator allow the observation of modulated Raman solvent and Rayleigh scattering, which are convenient for determining the zero reference phase.

INTRODUCTION

Fluorescence decay time measurements are essential to the understanding of nonradiative deexcitation processes in many physical and chemical systems (9) and to the interpretation of experiments using fluorophores as probes in biological systems, as, for example, in energy transfer processes and in the study of molecular dynamics and structural fluctuations of proteins and nucleic acids in solutions and in membranes (12).

Decay times can be measured either by the pulse-decay or by the phase-shift method (1). In the ideal pulse-decay measurement the fluorescence is excited with a light pulse that decays fast compared with the fluorescence; after the pulse termination, the time dependence of the fluorescence intensity decay is recorded either by direct or by sampling techniques. In the phase-shift method fluorescence is excited with amplitude-modulated continuous light, and the phase delay of the fluorescence modulation with respect to the phase of the exciting light modulation is measured. Single photon techniques can be incorporated into both methods to take advantage of improved signal-to-noise ratios realized for detecting weak signals when photomultiplier dark current is the dominant noise source (7,10). The two methods are formally equivalent if the phase delay is measured as a function of modulation frequency, since then the amplitude-time data and phase-frequency data are relatable by a Fourier transform. For experimental convenience, phase-shift measurements are usually obtained at only two or three frequencies, resulting in a very coarse sampling of the

phase-frequency function, but this is not a fundamental limitation. Thus, for example, sampling at perhaps 10 frequencies may be required to analyze data having multiple decay times (1,10), a common situation in biologically interesting cases such as non-equivalently bound fluorophores and anisotropic motion of fluorophores bound to macromolecules (4).

When the fluorescence decay times are less than a few nanoseconds, the pulse-decay method is experimentally difficult. Some of these difficulties and specific solutions to them have been described recently by Leskovar et al. (7) and Hartig et al. (3), including, for example, the need to deconvolve the exciting light pulse from the fluorescence response, the use of pulse amplifiers which push the limits of technology, and the effects of spurious system time delays. In contrast, the phase-shift method for measurement of subnanosecond decay times utilizes standard radio-frequency (RF) techniques and, because the phase-shift is independent of signal amplitude, it is relatively insensitive to exciting light source fluctuations.

In the phase-shift method, amplitude-modulated exciting light of intensity

$$I(t) = a_0 + \sum_{n=1}^{\infty} a_n \cos n\Omega t \quad a_0/\Sigma a_n \geq 1 \tag{1}$$

causes fluorescence of the form

$$F(t) = k\left(a_0 + \sum_{n=1}^{\infty} a_n \cos \delta_n \cos (n\Omega t - \delta_n)\right), \tag{2}$$

where Eq. 2 assumes negligible perturbation of the ground state population and a sample characterized by a single decay time. (See refs. 1 and 10 for considerations of multiple decay times.) $\operatorname{Tan} \delta_n = n\Omega\tau$ where $\Omega = 2\pi f$ and f is the fundamental modulation frequency, τ is the fluorescence decay time, and k is a constant including quantum efficiency, light collection efficiency, detector response, and receiver gain. At each frequency the excitation and fluorescence modulation depths are respectively $M_n = a_n/a_0$ and $m_n = a_n \cos \delta_n/a_0$. Thus $\cos \delta_n = m_n/M_n$. In principle the fluorescence decay time can be determined from a measurement of either the phase delay or the relative modulation depths.

As described below, narrow-band phase sensitive detection makes possible selection of the fluorescence response at a single frequency. Eq. 2 shows that the excitation modulation depth should be as large as possible to maximize the signal (proportional to $a_n \cos \delta_n$) with respect to the noise (proportional to $(a_0 + \Sigma a_n \cos \delta_n)^{1/2}$). The modulation frequency needs to be chosen so that both δ_n and $\cos \delta_n$ are measurable with reasonable integration times (in the order of seconds). For lifetimes between 0.05 and 10 ns, the modulation frequency needs to be in the range 100–20 MHz.

The main instrumental problem in phase-shift fluorometry is modulation of the exciting light. In the past the light was obtained from broad band source (e.g. xenon arc)-monochromator combinations and modulated by using either the Debye-Sears effect (2) (scattering of light by ultrasonic waves in a liquid medium) or the longi-

tudinal electrooptic effect (5,6). In principle the modulation frequency with electrooptic modulators can be varied continuously up to about 100 MHz, where heating of the crystal becomes substantial. However, the longitudinal devices require several kilovolts of applied modulating signal to achieve an experimentally useful modulation depth. Even with care taken to shield against RF leakage, these high levels still can cause interference with the detection system. In contrast, Debye-Sears devices require at most 100 V, but the modulation frequency can be varied only by changing the transducer or by driving it at odd harmonics of the fundamental, with corresponding decrease in developed acoustic power and consequent decrease in modulation depth. Furthermore Debye-Sears devices can present design problems at frequencies beyond 20 MHz.

In this paper we describe a phase-shift fluorometer that is basically a standard instrument, but technically different from fluorometers previously described and particularly easy to assemble. Its novel feature is laser illumination, which permits the use of a low-voltage (200 V) transverse mode electrooptic modulator. (For reasons described below, this type of modulator cannot be used with a conventional light source.) In addition it includes a simple field-effect transistor (FET) cascode RF amplifier at the photomultiplier tube output, a tuned PIN photodiode to obtain a phase reference signal, and a diode type double balanced mixer for phase-sensitive demodulation. The laser is not restrictive because tunable dye lasers are available. The circuits shown here are for fluorescence intensities suitable for analogue detection, but the detector system could be modified easily to the single photon phase-fluorometer described by Schlag et al. (10).

INSTRUMENT DESIGN

Fig. 1 is a schematic diagram of the system. The legend identifies the components. The basic design is similar to that described by others (see review by Birks and Munro [1]). With the exception of the PIN diode detector and cascode amplifiers, all components are commercial and used without modification. The assembly of the noncommercial amplifiers requires standard radio-frequency techniques with special guidelines provided by the transistor manufacturers. These amplifiers are small, battery powered, and can be exchanged easily with other similar amplifiers tuned to different frequencies. The transmitter accepts four crystals providing any frequency from 1 to 30 MHz. Considering Eqs. 4–6 below, usable modulation amplitude can be achieved even in the fourth harmonic of the fundamental modulator driving frequency. The upper frequency limit is the photomultiplier tube intrinsic frequency response. Although high frequency tubes (100 MHz) are available, their use means a compromise between frequency response and dark current noise.

Electrooptic modulators are crystals having an applied electric field-dependent birefringence (5,6). The most commonly used crystals are KDP (potassium dihydrogen phosphate) or ADP (ammonium dihydrogen phosphate). These uniaxial crystals become biaxially birefringent when an electric field is applied along the optic axis. The propagation velocity of light polarized along one principal axis of the polarizability tensor differs from that of light polarized along another optically inequivalent axis. With plane polarized incident light and proper crystal orientation, the propagation time phase delay causes light exiting from the crystal to have an applied field-dependent component polarized perpendicularly to the incident polarization. Light intensity passing through a polarization analyzer following the crystal then depends on the applied electric field.

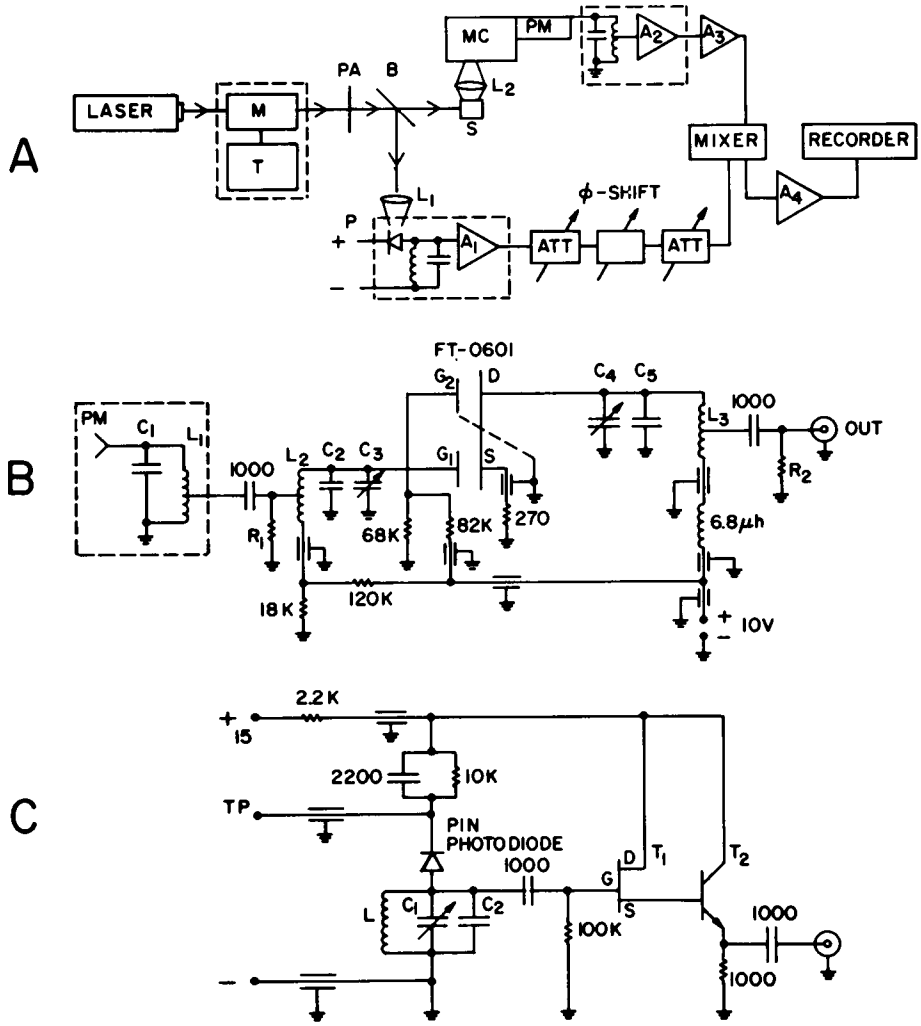

FIGURE 1 A) System block diagram. *M* is the Lasermetrics model 3078 transverse electrooptic modulator driven by a Heathkit DX-60B ham transmitter (Heath Company, Benton Harbor, Mich.), *T*. Light passes through a polarization analyzer, *PA* and is split by a beam splitter, *B*, with about 95% passing to the sample cuvette, *S*, and about 5% focused by lens L_1 onto the surface of the Hewlett-Packard model 5082-4220 PIN diode, *P*. Schematic for amplifier A_1 and the PIN diode tuning are shown in Fig. 1 C. The output of A_1 is fed through a variable attenuator, *ATT*, the variable phase shifter (Computer Devices, Inc., Burlington, Mass., model DV 580-1, 37 turn 29 ns. total delay line readable to ±0.01 turn. It was calibrated against propagation time delay obtained by varying the exciting beam-path length past the beam splitter.), another variable attenuator, and then to the reference input of a Hewlett-Packard 10534A double balanced mixer. Fluorescence imaged by L_2 onto the entrance slit of a Jarrel-Ash double monochromator, *MC*. The monochromator output is detected with an ITT-FW-130 photomultiplier tube, *PM*. The tuned photomultiplier load and amplifier A_2 schematics are shown in Fig. 1 B. Amplifier A_3 is a Hewlett-Packard 461A (broad band). A_4 is a Tektronix 1A7A amplifier (Tektronix, Inc., Beaverton, Ore.). Not shown in the block diagram is a tuned power amplifier (Hewlett-Packard 230B) following A_1 which rejects unwanted harmonics of the modulated intensity and provides the 1.0 V rms necessary to operate the mixer.

The optical phase delay between rays polarized along two inequivalent optical axes is proportional to (EL/λ) where E is the applied electric field intensity, L the crystal length, and λ the optical wavelength. In a longitudinal device, E is parallel to the light propagation direction; the electric field intensity is proportional to V/L where V is the applied voltage; the optical phase delay is then proportional to V. The magnitude of the delay is determined by the material electrooptic coefficient. In a longitudinal device, the voltage required to produce half-wave retardation, i.e. rotation of the incident polarization plane by 90°, is in the order of 10 kV. (90° rotation is required for maximum modulation index.) In a transverse device, E is *transverse* to the light propagation direction; the electric field is proportional to V/t, where t is the crystal thickness; the phase delay is then proportional to VL/t. Thus long thin crystals reduce the required driving voltage. However, to reduce the driving voltage to a few hundred volts, the crystals have to be so long and thin that the incident beam diameter and angular divergence must be very small. Such a beam is available only from a laser.

We use a KDP transverse modulator (model 3078FX, Lasermetrics, Inc., Teaneck, N.J.) and the 441.6 nm line of a helium-cadmium laser. At this wavelength the modulator half-wave retardation voltage is 200 V obtained from the transmitter without modification of the output tank circuits. The modulator also functions, with only small increase in insertion loss, at 325 nm, obtainable from the same laser.

With vertically polarized incident light, the intensity passing through a horizontally oriented analyzer is proportional to $(1 - \cos(\pi V(t)/V_0))$, where $V(t)$ is the modulating signal and V_o is the half-wave retardation voltage. For modulation of the form $V(t) = V_o(A + B\cos\Omega t)/2$ the power passing the analyzer is, to within a constant factor,

$$P = 1 - \cos\alpha[J_0(\beta) - 2J_2(\beta)\cos(2\Omega t) + 2J_4(\beta)\cos 4\Omega t - 2J_6(\beta)\cos 6\Omega t + \ldots]$$
$$+ \sin\alpha[2J_1(\beta)\cos\Omega t - 2J_3(\beta)\cos(3\Omega t) + 2J_5(\beta)\cos(5\Omega t) - \ldots] \quad (4)$$

where $\alpha = \pi A/2$; $\beta = \pi B/2$ and $J_n(x)$ are ordinary Bessel functions. For the special case of $A = 0$ and $B = 1$

$$P \cong 1 - [-0.305 - 0.97\cos(2\Omega t) + 0.31\cos(4\Omega t) - 0.05\cos(6\Omega t)] \quad (5)$$

and when $A = 1$ and $B = 1$ (constant field bias applied to the crystal)

$$P \cong 1 - [1.14\cos(\Omega t) - 0.14\cos(3\Omega t) + 0.01\cos(5\Omega t)] \quad (6)$$

Experimentally the preferred operation mode is with $A = 0$ because the frequency of the maximum modulated intensity is 2Ω rather than Ω. Thus the receiver is tuned to twice the transmitter output frequency and pickup of stray RF radiation at the transmitter frequency is minimized.

The modulated fluorescence is detected by an uncooled photomultiplier tube (ITT FW-130, ITT, Electro-Optical Products Div., Roanoke, Va.) at the output of a Jarrel-Ash 75 cm double monochromator (Jarrel Ash Div., Fisher-Scientific Co., Waltham, Mass.). The double mono-

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B). Cascode tuned amplifier schematic. The values of unspecified components are chosen to give maximum stable output at the desired frequency. They depend on the frequency and stray capacitance. Indicated capacitances are in picofarads and resistances in ohms. FT-0601 is a Fairchild RF dual-gate metal oxide semiconductor FET (Fairchild Industries, Winston-Salem, N.C.). The various sections are isolated and connected via 1,000 pF feed through capacitors. At 28.8 MHz, the bench-measured voltage gain was 15 and the Q about 30.

C) Tuned PIN diode-amplifier schematic. The values of unspecified components are as in Fig. 1 B. T_1 is a 2N4416 and T_2 a 2N3904. The PIN diode is Hewlett-Packard model 5082-4220.

chromator is not necessary for fluorometry, but it maximizes rejection of elastically scattered exciting light and allows detection of modulated solvent Raman scattering useful in calibrating the instrumental phase delay. The photomultiplier load is a tuned transformer link adjusted to provide stable impedance matching to the input of the FET cascode tuned amplifier shown in the schematic of Fig. 1 B.

The reference voltage for the mixer is obtained with a PIN photodiode tuned amplifier combination (Fig. 1 C) to detect a few percent of the exciting light directed by a beam splitter through a focusing lens onto the PIN photodiode surface. It was followed by a tuned power amplifier, (model 230B, Hewlett-Packard Co., Palo Alto, Calif.) to obtain the 1.0 V root mean square (RMS) required to operate the mixer. The tuning was sufficiently sharp that the output at unwanted harmonics of the modulated intensity was below the level of detectability.

The quantity $k \cos \delta$ (see Eqs. 1 and 2) is determined from the ratio of the maximum fluorescence and the maximum Rayleigh or Raman intensities obtained by adjusting the phase shifter to yield the respective maximum signals. The constant k is given by the ratio of these two signal intensities in the absence of modulation. This latter measurement used conventional photon counting instrumentation, but for most fluorescence and Rayleigh signals it could be done by measuring directly the average voltage developed across a photomultiplier tube load resistor.

The possibility of wavelength-dependent phase shifts caused by photon energy-dependent photo electron transit times has been pointed out by several workers (7,8). The modulated Raman spectrum of benzene enables a check of this over a wavelength range of about 500 Å. Since the benzene Raman spectrum has a high frequency fundamental at 3,061 cm^{-1} to lower energy of the Rayleigh scattering, any wavelength-dependent phase shift would cause the relative intensity distribution in the modulated Raman spectrum to differ from that of the unmodulated spectrum. We observed identical relative intensities in the modulated and unmodulated spectra. Wavelength-dependent phase shifts could still occur at longer wavelengths, but the decay time measurements here were all done within the above range.

INSTRUMENT PERFORMANCE

The instrument performance is illustrated here with data from fluorescein with 28.8 MHz modulation frequency. At 42.5 MHz, the frequency response of the FW-130 photomultiplier tube is down by at least 6 dB from that at 28.8 MHz. The most convenient way to determine the phase shift of the fluorescence with respect to undelayed elastic or Raman scattering is to determine the phase shifter setting at which the signal and reference are 90° out of phase and the output of the phase detector is a null. Around the null, the mixer output is approximately proportional to the phase angle difference between the signal and the reference. Trace A in Fig. 2 shows the residual unbalance of the phase detector output from fluorescein (10 μg/ml) fluorescence as the phase shifter was moved through the null. The modulated fluorescence peak photon rate was in the order of 10^4 photons/s obtained with a 0.02 mW laser power and a 5 cm^{-1} spectral slit width, sufficiently narrow for recording well-resolved Raman spectra. The maximum phase detector output obtained with the reference in phase with the signal was about 0.2 mV. The approximate noise level, dominated by shot noise proportional to the square root of the fluorescence intensity, shown in trace B of Fig. 2 was about 0.01 mV with a time constant of a few seconds. Thus the fluorescence signal-to-noise ratio was in the order of 20. Although this could be improved by decreasing the attenuation of the laser power, it gives an estimate of the system performance in the presence of noise. The linear plot in Fig. 2 C shows that the small phase angle approximation is experimentally realized. The error bars are approxi-

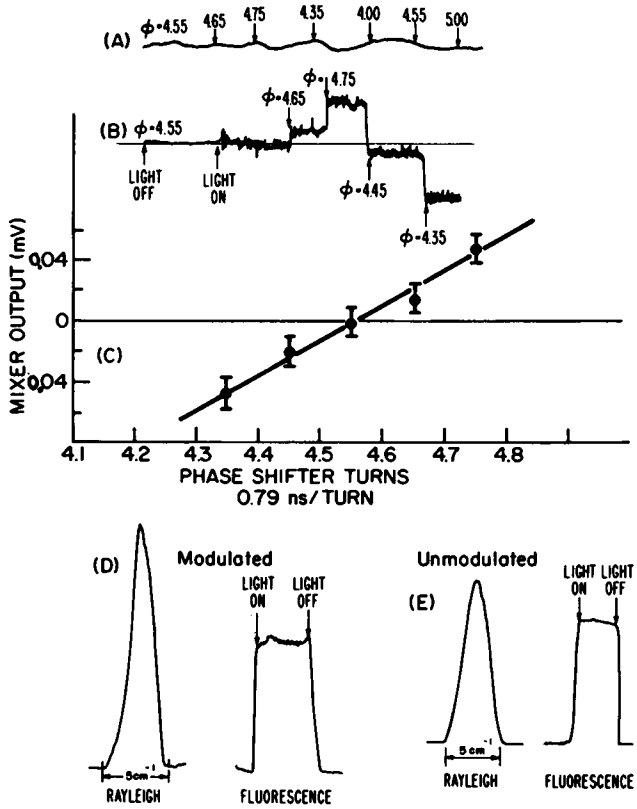

FIGURE 2 A Residual phase detector unbalance determined by blocking monochromator entrance and recording phase detector output at various settings of the phase shifter. Numerical values of ϕ correspond to the phase shifter position expressed in turns from one extreme. B. Phase detector output for modulated fluorescein (10 μg/ml) fluorescence at various values of phase shifter setting near 90° out of phase with respect to the signal. Modulation frequency is 28.8 MHz. C. Phase detector output of Fig. 2 B illustrating approximate linear dependence of signal amplitude on phase angle when reference and signal are 90° out of phase. D. Maximum amplitudes of modulated Rayleigh and fluorescein (10 μg/ml) fluorescence signals obtained by adjusting phase shifter to yield maximum signal. The Rayleigh signal was obtained by scanning the monochromator through the Rayleigh line. Its line width is much less than the spectral slit width; thus it is broadened to the order of the 5 cm^{-1} spectral slit width. The fluorescence was measured with the monochromator set at 19,500 cm^{-1}. The output amplifier gain was the same for both signals. E. Signal amplitudes as in Fig. 2 D but with modulation off. Signal was detected by conventional photon counting. From Eqs. 1 and 2 in text, $\cos\delta = (F_m R)/(F R_m)$ where F_m and R_m are maximum amplitudes of modulated fluorescence and Rayleigh signals and F and R are the corresponding unmodulated signal amplitudes. From the data in Fig. 2 D and 2 E, $\cos\delta = 0.79$, corresponding to $\tau = 4.25$ ns.

mately plus-minus the rms noise inherent in the signals for each setting of the phase shifter.

Table I shows measured decay times of potassium iodide-quenched fluorescein fluorescence together with those measured by Spencer and Weber (11). The uncertainties are estimated from the uncertainty in determining the true zero signal in the presence of the noise, as in Fig. 2 C, and from the ±0.01 turn uncertainty in reading

TABLE I

POTASSIUM IODIDE QUENCHING OF FLUORESCEIN* FLUORESCENCE

KI	τ	τ (ref. 8)
M	*ns*	*ns*
0.00	4.23 ± 0.02	4.70 ± 0.03
0.10	2.50 ± 0.03	2.26 ± 0.03
0.19	1.65 ± 0.04	1.40 ± 0.03
0.48	0.43 ± 0.05	0.49 ± 0.03

*10 μg/ml in 0.01 M NaOH.

the phase shifter. The experimental uncertainty in our measurement increases for a given integration time as the signal-to-noise ratio decreases with the increasing quenching. Our main interest here was in demonstrating the precision of our instrument and its capability for subnanosecond measurements. The 10% discrepancies between our absolute values and those of Spencer and Weber probably are not important in view of the concentration dependence of fluorescein decay time cited by Spencer and Weber and considering that we did not purify the samples or purge oxygen from them.

Fig. 2 D and 2 E show the determination of the fluorescein (10 μg/ml) decay time from the measurement of relative modulation depths. The expression shown in the caption follows from Eqs. 1 and 2. The decay time thus obtained agrees with that obtained from the phase-shift measurement.

Received for publication 26 May 1977.

REFERENCES

1. BIRKS, J. B., and I. H. MUNRO. 1967. The fluorescence lifetimes of aromatic molecules. *Prog. React. Kinet.* **4**:239–303.
2. BORN, M., and E. WOLF. 1964. Principles of Optics. Macmillan Publishing Co., New York.
3. HARTIG, P. R., K. SAUER, C. C. LO, and B. LESKOVAR. 1976. Measurement of very short fluorescence lifetimes by single-photon counting. *Rev. Sci. Instrum.* **47**:1122–1129.
4. ISENBERG, I., and R. DYSON. 1969. The analysis of fluorescence decay by a method of moments. *Biophys. J.* **9**:1337–1350.
5. JONA, F., and G. SHIRANE. 1962. Ferroelectric Crystals. Macmillan Publishing Co., New York. 82–87.
6. KAMINOW, I. P., and E. H. TURNER. 1966. Electrooptic light modulators. *Proc. IEEE (Inst. Electr. Electron Eng.).* **54**:1374–1390.
7. LESKOVAR, B., C. C. LO, P. R. HARTIG, and K. SAUER. 1976. Photon counting system for subnanosecond fluorescence lifetime measurements. *Rev. Sci. Instrum.* **47**:1113–1121.
8. MULLER, A., R. LUMRY, and H. KIKUBUN. 1965. High performance phase fluorometer constructed from commercial subunits. *Rev. Sci. Instrum.* **36**:1214–1226.
9. SCHLAG, E. W., S. SCHNEIDER, and S. F. FISCHER. 1971. Lifetimes in excited states. *Annu. Rev. Phys. Chem.* **22**:465–526.
10. SCHLAG, E. W., H. L. SELZLE, S. SCHNEIDER, and J. G. LARSEN. 1974. Single photon phase fluorimetry with nanosecond resolution. *Rev. Sci. Instrum.* **45**:364–367.
11. SPENCER, R. D., and G. WEBER. 1969. Measurements of subnanosecond fluorescence lifetimes with a cross-correlation phase fluorometer. *Ann. N.Y. Acad. Sci.* **158**:361–376.
12. THAER, A. A., and M. SERNETZ. 1973. Fluorescence Techniques in Cell Biology. Springer-Verlag N.Y. Inc., New York.