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Construction of a Nanosecond Fluorometric System for Applications to Biological Samples at Cell or Tissue Levels

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A fluorometric system utilizing the single photon counting technique which records the decay of the intensity and the anisotropy of fluorescence following pulse excitation is described. Both macroscopic observations on cellular suspensions and measurements on individual cells under an optical microscope are possible. The system is so designed to permit precise evaluation of complex anisotropy decays, which is essential for studies of cells or tissues. Data are processed by a method which properly analyzes these complex decays. A preliminary study on erythrocyte membrane has confirmed the high resolution and precision of the system.

§1. Introduction

Measurement of the decay of fluorescence, or the so-called nanosecond fluorometry, has been proved to be a useful means in the fields of biophysics and biochemistry.[1-4] Thus the decay of fluorescence intensity, or the fluorescence lifetime, characterizes the polarity of the environment of fluorescent molecules, while the decay of fluorescence anisotropy provides information about the rotational motion and/or the spatial distribution of the molecules.[1,5]

Biological applications of this technique have been confined mostly to the studies of relatively simple systems such as proteins or nucleic acids in solutions. Although fluorometric studies on higher systems as cellular membranes or muscles are abundant in the literature,[6] most, if not all, have adopted the steady-state excitation. However, it is on these latter systems that the nanosecond fluorometry will exhibit its full advantages, because only the time-dependent measurement can distinguish the complex decays expected in these samples. This is particularly true of the anisotropy decay, for which the time-dependent study is almost a necessity for the correct understanding of underlying phenomena.

Nanosecond fluorometry on systems of higher levels requires some refinements of the technique. Firstly, it will be necessary to carry out both the macroscopic and microscopic observations. The macroscopic measurements of cellular suspensions yield precise information under well regulated external conditions, while the fluorometry under a microscope enables the *in vivo* studies of living tissues, or the discrimination of individual cells.

Secondly, the expected complexity of the decay requires a measuring system having a good time resolution as well as a high precision. The obtained data should be properly analyzed to include this complexity.

Thirdly, the effect of the inevitable turbidity of the samples should be minimized by the use of an appropriate optics. When suspensions are examined, the sedimentation of the material during a measurement should also be avoided.

In this paper, a fluorometric system is described which meets all the requirements stated above. It records the decay of the intensity and the anisotropy of fluorescence in the nanosecond region according to the single photon counting technique.[7] For convenience, the system is named "nanosecond fluorometer" when assembled to perform the macroscopic observations, while it is called "nanosecond microfluorometer" when combined with an optical microscope.

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Each fluorometer is described in §2 and §3. Section 4 deals with the analysis of the decay data obtained. Results of preliminary measurements on erythrocyte membrane are presented in §5, upon which the performance of the present fluorometric system is discussed in §6.

§2. Nanosecond Fluorometer

The nanosecond fluorometer described here is an apparatus which excites fluorescence by light pulses of duration a few nanoseconds and digitally records the decay of both the intensity and the anisotropy of emitted fluorescence.

A simplified block diagram is shown in Fig. 1. The excitation source (L) is a free-running discharge lamp of coaxial type. The original design of Hundley *et al.*[8] has been modified as follows: spring contacts have been adopted instead of soldering to allow the easy reconstruction; the electrical signal coincident with the discharge is picked up by a small antenna coil in the bottom of the lamp, thus isolating the detection circuit from the discharge current; resistors are of high wattage type to allow a larger average current. The lamp is filled with either hydrogen or nitrogen at a pressure of a few to ten atmospheres.

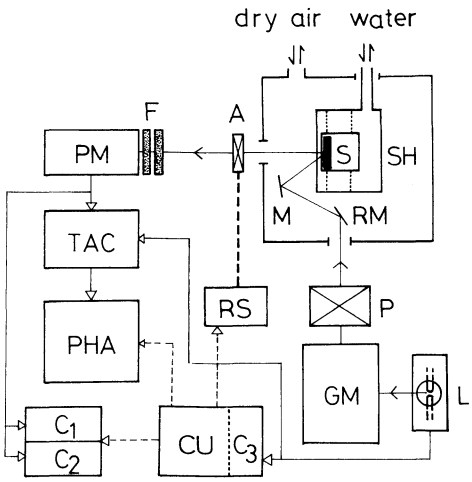

Fig. 1. Block diagram of the nanosecond fluorometer. L: light source, GM: monochromator, P: polarizer, RM: removable mirror, M: mirror, S: sample in quartz cuvette, SH: sample holder, A: analyzer, RS: rotary solenoid, F: filters, PM: photomultiplier tube, TAC: time-to-amplitude converter, PHA: multi-channel pulse height analyzer, C1 and C2: dual pulse counter, CU: control unit, C3: built-in pulse counter.

The hydrogen lamp was found to be superior than the nitrogen lamp in several points. Firstly, its light pulse does not trail any tail, which would otherwise distort the measured decay of fluorescence (compare Fig. 3 and 4). Secondly, the spectral output is continuous from ultraviolet to over 600 nm, and the waveform of the output pulse does not depend appreciably on wavelength at least in the range 320–500 nm except at 486 nm, the characteristic line of hydrogen.* This is an important feature which enables the correct analysis of fast decays, as will be discussed in later sections.

The nitrogen lamp is used when the fluorescence of the sample is very weak, because the intensity is an order of magnitude higher than the hydrogen lamp. An air lamp, most commonly used by other workers, was found to be inferior to the hydrogen lamp: the intensity is low and the spectral output is confined to several lines having different waveforms.

The light from the source is passed through a grating monochromator GM (Riko Tsusho MC-30), polarized vertically by a Glan prism P, and lead to a sample S in a 2×10 mm quartz cuvette. Figure 1 shows the case of surface optics in which the sample is excited through the surface of detection. The ordinary 90° optics is also possible by the removal of RM.

The temperature of the sample is controlled by the circulation of water to within 0.2°C, and is continuously monitored with a tip thermister. Dew condensation at low temperatures is prevented by the circulation of dry air. The sample can be agitated by a small magnetic tip in the cuvette, which is driven by a built-in magnetic stirrer.

The fluorescence emission is analyzed by a sheet polarizer A (Polaroid HNP'B), which is rotated by a rotary solenoid between the vertical and horizontal orientations; two stoppers act as electric contacts which report the proper orientations.

The emission wavelength is set either by filters F or a prism monochromator (Zeiss PMQ II). The light is focused onto a small portion of the photocathode of a photomultiplier tube PM (RCA 8850), thus eliminating the possible time jitter.[9]

The electrical signals are processed as in the

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usual single photon counting technique.[7] Thus the time difference between the discharge of the lamp and the detection of a single photon of fluorescence by the photomultiplier tube is converted to a voltage pulse by a time-to-amplitude converter TAC (Ortec 437A). The amplitude of the pulse is digitized and stored by a multi-channel pulse height analyzer PHA (Northern Scientific NS-600). Usually the 1024-channel memory of the PHA is divided into halves, each corresponding to the vertical or horizontal position of the analyzer A. After 10^6 to 10^8 excitations, the number of counts accumulated in each memory channel of the PHA versus pulse height gives the decay time course of the fluorescence. The total numbers of photons detected are monitored by a dual counter C1 and C2.

The operation of the whole system is governed by a control unit CU. For every 10^5 excitations the unit commands the rotation of the analyzer A, and at the same time selects a proper half of the memory of the PHA and a proper one of the dual counter. The measurement is interrupted until the analyzer settles down in the assigned position.

The start and the stop of a measurement as well as the readout of the data (through a teletypewriter) is commanded by the control unit. The intermediary data can be automatically printed or punched on a paper tape at present intervals. The unit can be directly connected to a minicomputer (Hitac 10), which enables the real time data processing.

The static fluorometry under the condition of steady-state excitation is also possible by replacing the excitation source with a 500-W xenon lamp. In this case the fluorescence intensity is measured as the number of photons detected per unit time interval. In particular, the multichannel scaling capability of the PHA enables the digital recording (and averaging) of transient changes in the intensity and/or the anisotropy of fluorescence following *e.g.* the addition of chemically active substances. Due to the high sensitivity of the photon counting, the excitation intensity can be reduced to a very low level so that the effect of any photodegradation of fluorophores is neglected.

§3. Nanosecond Microfluorometer

The electric part of the system described in the preceding section can be combined with an Olympus XMSP optical microscope to construct the nanosecond microfluorometer.

The optical diagram is shown in Fig. 2. The light emitted by the discharge lamp is monochromatized by a set of filters and focused on a pin-hole. A reflecting condenser lens (Olympus MO30, 30× and NA0.46−0.16, or MO90, 90× and NA0.65−0.16) gathers the passed light and forms the image of the pin-hole on a sample between a quartz slide and a quartz cover slip. The diameter of the light spot at the sample can be varied down to 1 μm by changing the size of the pin-hole to 0.2 mm at the magnification of the condenser of 30×. The fluorescence emitted by the sample is collected by an objective congruent with the condenser, passed through barrier filters and finally detected by the RCA 8850 photomultiplier tube.

A pair of sheet polarizers (Polaroid HNP'B) are used in the anisotropy measurements. Thanks to the relatively large working distance of the reflection lenses (about 7 mm at the magnification of 30×), the polarizers can be inserted just above and below the sample. This assures the accurate determination of the anisotropy free from the depolarizing effect of lenses. Both the polarizer and the analyzer may be set at any desired angles to facilitate the examination of optically anisotropic samples.

Usually an accurate measurement tahes one to a few hours. When a cell suspension is examined, it is kept from evaporation in a way depicted in Fig. 2. A drop of the suspension is applied under a cover slip, which is mounted on a hollow slide. The space between them is filled with the same suspension and sealed with liquid paraffin from outside.

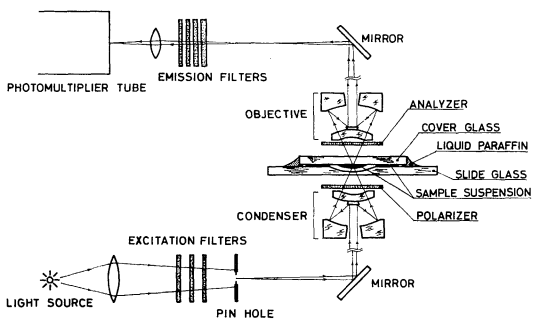

Fig. 2. Optical diagram of the nanosecond microfluorometer.

§4. Data Analysis

Data accumulated in the pulse height analyzer are usually punched on paper tape and processed by a large computer (Hitac 8800/8700) to obtain several time constants which characterize the sample.

A data set consists of $I_{//}(t)$, $I_{\perp}(t)$ and $P(t)$, where $I_{//}(t)$ and $I_{\perp}(t)$ are the time courses of fluorescence intensity component polarized parallel and perpendicular to the direction of polarization of the excitation light, and $P(t)$ is the time course of the excitation light pulse taken by placing an aluminum mirror in the sample holder. Usually $P(t)$ is measured in duplicate, at the beginning and the end, in order to check the absence of any drift.

The observed time courses are first corrected for photon pile-up;[10] then the background counts corresponding to the dark noise of the photomultiplier tube are subtracted. The latter is calculated as the average count in the part before the rising phase of each time course.

Fluorescence intensities are then corrected for the difference in the sensitivity of the detection system as to the direction of polarization of the incident light. In the case of the nanosecond fluorometer, $I_{\perp}(t)$ is multiplied by the sensitivity ratio determined by a separate measurement in which the sample is excited with horizontally polarized light through 90° optics.[11] When the microscope is used, the ratio is calculated from the following equation:

$$[I^c_{//}/I^c_{\perp}]^2=[I_{xx}/I_{xy}][I_{yy}/I_{yx}], \tag{1}$$

where the superscript c denotes the corrected quantities and I_{xy}, for example, represents the fluorescence component taken with the polarizer and the analyzer in the x- and y-derections, respectively, where x and y are the arbitrary chosen rectangular axes in the plane of the microscope stage. This equation applies only when the sample in the light spot is isotropic as a whole. With anisotropic samples, correction by means of a standard sample is required.

From $I_{//}(t)$ and $I_{\perp}(t)$ corrected as above, the quantities which characterize the fluorescence of the sample are calculated as follows:

$$I_{\mathrm{T}}(t)=I^c_{//}(t)+2I^c_{\perp}(t), \tag{2}$$

$$I_{\mathrm{D}}(t)=I^c_{//}(t)-I^c_{\perp}(t), \tag{3}$$

$$r(t)=I_{\mathrm{D}}(t)/I_{\mathrm{T}}(t). \tag{4}$$

The quantity $I_{\mathrm{T}}(t)$ is proportional to the total fluorescence intensity; $r(t)$ is the anisotropy which is a measure of the degree of polarization of fluorescence. In the following section the experimental results are shown in terms of $I_{\mathrm{T}}(t)$ and $r(t)$.

As can be seen in Figs. 3–5, $I_{\mathrm{T}}(t)$ and $r(t)$ defined above are the responses to the excitation pulse $P(t)$ whose duration is finite. These quantities are related to $I^{\delta}_{\mathrm{T}}(t)$ and $r^{\delta}(t)$, the responses to a truly impulsive excitation expressed as $\delta(t)$, Dirac's delta function, by the following convolution equations:

$$I_{\mathrm{T}}(t)=\int_0^t P(t')I^{\delta}_{\mathrm{T}}(t-t')\,\mathrm{d}t', \tag{5}$$

$$I_{\mathrm{D}}(t)=\int P(t')I^{\delta}_{\mathrm{T}}(t-t')r^{\delta}(t-t')\,\mathrm{d}t'. \tag{6}$$

The quantities $r^{\delta}(t)$ and $I^{\delta}_{\mathrm{T}}(t)$ can be reproduced most satisfactorily by the following curve fitting procedure. First, they are assumed to be expressed as sums of exponentials:

$$I^{\delta}_{\mathrm{T}}(t)=\sum_{i=1}^{N}\alpha_i\exp(-t/\tau_i), \tag{7}$$

$$r^{\delta}(t)=\sum_{i=1}^{N'}\beta_i\exp(-t/\varphi_i), \tag{8}$$

where α_i, β_i, τ_i and φ_i are the parameters to be determined; in particular τ_i is considered to be the fluorescence lifetime of the i-th component.

The values of these parameters are so determined as to minimize the following quantities:

$$F_{\mathrm{T}}\equiv\sum_{t_n}\{[I^{\mathrm{obs}}_{\mathrm{T}}(t_n)-I^{\mathrm{calc}}_{\mathrm{T}}(t_n)]^2/I^{\mathrm{obs}}_{\mathrm{T}}(t_n)\}, \tag{9}$$

$$F_{\mathrm{D}}\equiv\sum_{t_n}\{[I^{\mathrm{obs}}_{\mathrm{D}}(t_n)-I^{\mathrm{calc}}_{\mathrm{D}}(t_n)]^2/I^{\mathrm{obs}}_{\mathrm{D}}(t_n)\}. \tag{10}$$

That is, the observed time courses are fitted with the calculated convolutions:

$$I^{\mathrm{calc}}_{\mathrm{T}}(t_n)=\{\sum_{t'_n=0}^{t_n}P(t'_n)I^{\delta}_{\mathrm{T}}(t_n-t'_n)\}-I^{\delta}_{\mathrm{T}}(0)C(t_n), \tag{11}$$

$$I^{\mathrm{calc}}_{\mathrm{D}}(t_n)=\{\sum_{t'_n=0}^{t_n}P(t'_n)I^{\delta}_{\mathrm{T}}(t_n-t'_n)r^{\delta}(t_n-t'_n)\}-I^{\delta}_{\mathrm{T}}(0)r^{\delta}(0)C(t_n). \tag{12}$$

Here the integrals in eqs. (5) and (6) have been approximated by summations with a correction factor $C(t_n)$. This factor is chosen as

$$C(t_n)=\frac{1}{2}P(t_n)+\frac{1}{24}[P(t_{n+1})-P(t_{n-1})], \tag{13}$$

on the assumption that $P(t)$ is linear in t within

the interval of $t_{n-1} \leqq t \leqq t_{n+1}$.

The denominators in eqs. (9) and (10) are the weight factors which compensate the statistical fluctuation in the number of detected photons (expected to be proportional to the square root of the number of counts).

The minimization of F_T and F_D is effected according to the FORTRAN program of Fletcher and Powell.[12] The calculation is repeated for $N=1, 2, 3$ until a good enough fit (judged by the value of F or by eye) is obtained. Usually the calculation takes several seconds for $N=2$.

Analysis of fast decays requires special precautions. Thus the wavelength dependence of the response characteristics of a photomultiplier tube cannot be neglected when decay time constant less than a few nanoseconds is determined.[13] However, since the output waveform of our hydrogen lamp is practically independent of wavelength, as stated before, it is possible to eliminate the above effect by simply measuring $P(t)$ at the wavelength of fluorescence emission. Also the width of the time window $\Delta t \equiv t_n - t_{n-1}$ must be small as compared to the time constants involved.

§5. Results of Observations

In order to test the performance of the fluorometers, fluorescence decays of 1-anilino-8-naphthalene sulfonate (ANS) adsorbed on erythrocyte membrane (ghost) have been measured. Both macroscopic and microscopic measurements are possible on this sample. Since the dye ANS is non-fluorescent in water and fluoresces strongly when adsorbed on ghost,[14] the observed fluorescence decays reflect the nature of ANS binding sites in the membrane.

Figure 3 shows the results of a macroscopic measurement by the nanosecond fluorometer. The thick broken line is $P(t)$ of the hydrogen lamp. The dots represent the measured decay of total fluorescence intensity $I_T(t)$. The thin broken line superposed on dots is the calculated best fit (eq. (11)) in which a single lifetime τ is assumed. Clearly it deviates from the observed time course. A satisfactory fit (solid line) was obtained on the assumption of two lifetimes:

$$I_T^{\delta}(t) \propto 0.56 \exp(-t/4.9) + 0.44 \exp(-t/13.2), \tag{14}$$

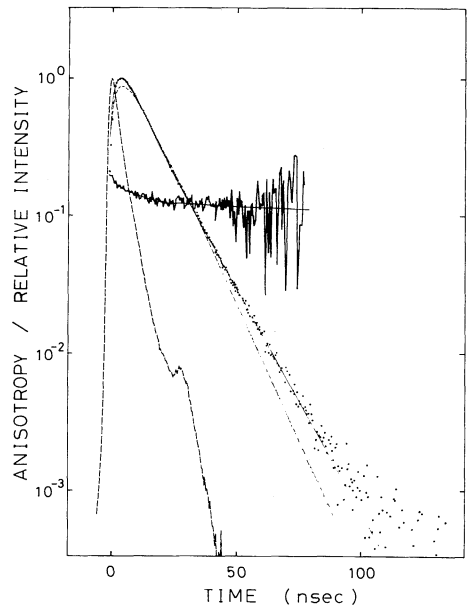

Fig. 3. Fluorescence decay of ANS adsorbed on bovine erythrocyte ghost. Macroscopic measurement by the nanosecond fluorometer. Thick broken line: excitation light pulse $P(t)$ (hydrogen, 8 atm), dots: total fluorescence intensity $I_T(t)$, zigzag line: fluorescence anisotropy $r(t)$, thin broken and solid lines: calculated best fit curves assuming one and two time constants, respectively. Ghost was prepared according to Dodge *et al.*[15] [ANS]=1.67 mM, [ghost]=2.1 mg dry wt./ml, in isotonic NaCl. Excitation at 380 nm through surface optics, emission through Toshiba UV-39 and Corning CS3–72 filters.

where unit of time is nanosecond. Thus at least two different sets of ANS binding site exist in erythrocyte ghost.

The zigzag line in the figure is the fluorescence anisotropy $r(t)$ defined in eq. (4). Despite the fluctuations at large t, the figure shows that r becomes almost constant at $t \gtrsim 20$ nsec. This indicates that the rotational motion of ANS molecules in the membrane is restricted within an angle, at least in the nanosecond range. The best two-exponential fit (thin solid line) was obtained with $\phi_1 = 5.0$ nsec and $\phi_2 = 905$ nsec, the former corresponding to the fast decay seen in the figure. This fast decay most probably results from the excitation energy transfer between ANS molecules, as will be discussed elsewhere.

A similar sample was examined under the nanosecond microfluorometer and the results are shown in Fig. 4. About 100 ghosts were

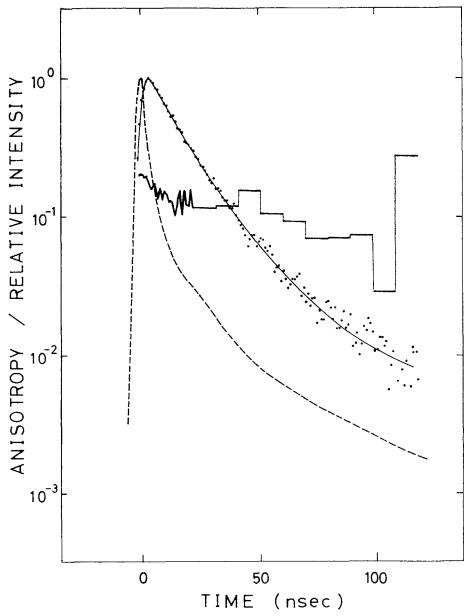

Fig. 4. Fluorescence decay of ANS adsorbed on bovine erythrocyte ghost. Microscopic measurement by the nanosecond microfluorometer. Broken line: $P(t)$ (nitrogen, 4 atm), dots: $I_T(t)$, zigzag line: $r(t)$, thin solid line: calculated best fit curve assuming two lifetimes. [ANS]=1.0 mM. Excitation through Corning CS7–37 and Schott UG-1, emission through Toshiba UV-39 and Corning CS3–72 filters. Diameter of the light spot=26.6 μm.

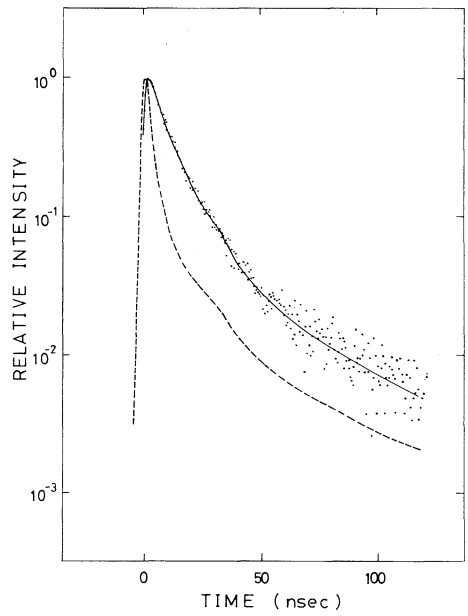

Fig. 5. Fluorescence decay of ANS adsorbed on bovine erythrocyte ghost. Microscopic measurement without polarizers. Broken line: $P(t)$ (nitrogen, 5 atm), dots: $I_T(t)$, solid line: calculated best fit curve assuming two lifetimes. [ANS]=2.5 mM. Flters were same as in Fig. 4. Diameter of the light spot=13.3 μm.

included in the light spot of diameter 26.6 μm. The excitation filters were Corning CS7-37 and Schott UG-1 (broad band around 360 nm), and the emission filters were Toshiba UV-39 and Corning CS3-72. This combination shuts out the excitation light almost perfectly.

Although $I_T(t)$ is severely distorted by the long tail of the nitrogen pulse, two-exponential analysis yielded

$$I_T^{\delta}(t) \propto 0.52 \exp(-t/5.7) + 0.48 \exp(-t/13.4), \tag{15}$$

which is in reasonable agreement with eq. (14).

The fluctuation of $r(t)$ is larger than in Fig. 3, because of the insufficient accumulation due to very weak fluorescence (peak count of I_T about 5000). Although precise analysis is difficult, the time course of $r(t)$ is seen to be similar to that in Fig. 3: fast decay with a time constant several nanoseconds followed by an almost flat phase.

The sensitivity of the microfluorometer is increased by an order of magnitude when the polarizer and the analyzer are omitted. Figure 5 shows the decay of fluorescence intensity emitted by about 10 ghosts in a light spot of 13.3 μm^{ϕ}. The filter combination was the same as in Fig. 4.

Here, too, the decay is not a simple exponential and the best fit (solid line) could be obtained by putting

$$I_T^{\delta}(t) \propto 0.68 \exp(-t/1.3) + 0.32 \exp(-t/7.4). \tag{16}$$

The values of τ are considerably smaller than in eq. (14) or (15). Under the condition of 10 ghosts in the light spot, the quantity of free ANS far exceeds the bound one. Therefore, the shorter lifetime can be attributed to that of ANS in the medium. In fact, the clear medium obtained by centrifugation gave a lifetime of 1.2 nsec (this value is larger than that of ANS in water and may indicate the presence of some solubilized material). The longer lifetime of 7.4 nsec corresponds to the average lifetime of membrane-bound ANS, from which the properties of ANS-binding sites can be inferred.

§6. Discussion

In macroscopic measurements, the greatest

merit of the single photon counting technique is that it enables the precise recording of a decay time course over several orders of magnitude; thus complex decays are readily distinguished. The adoption of the hydrogen lamp in our system strengthens this advantage by reducing the effect of trailing tail of the excitation pulse. Measured decays are almost identical with the response to $\delta(t)$=excitation except in the vicinity of the peak.

In Fig. 3, for example, we can see without the aid of computer analysis that the anisotropy r decays with a time constant a few nanoseconds and then remains at a constant value. Note that this constancy and its value can be correctly observed only with the combination of the sharp excitation pulse and the wide dynamic range of the detection system. This pattern of the anisotropy change actually is a common feature of various membrane systems, and often the constant value of r is a good measure of the degree of orientation of the dye molecules in the membrane.*

On the other hand, the hydrogen lamp also enables the accurate determination of fast decay constants, as has been discussed in the previous sections. This is difficult with the common air lamp, because its output waveform varies at different wavelengths. This fast decay gives us information about the speed of rotational motion and/or the spatial distribution of the molecules.

Thus the nanosecond fluorometer described here is ideal for the analysis of complex systems as membrane where both the fast decay and the following stationary phase are important.

The correct determination of $r(t)$ requires that the relative amplitude of $I_{//}(t)$ and $I_{\perp}(t)$ be properly measured. Since a precise measurement with the single photon counting technique may take a few hours, separate measurements of $I_{//}(t)$ and $I_{\perp}(t)$ will not give the proper ratio, due to the possible drift of the excitation intensity. The present fluorometer solves this problem by measuring $I_{//}(t)$ and $I_{\perp}(t)$ alternatively.

Samples like cell suspensions usually scatters light substantially. Therefore, a long path length of either the excitation or emitted light in the sample results in the apparently small anisotropy. In addition, when the sample contains natural chromophores as with intact erythrocyte, or when the external dye concentration is high, the excitation light is absorbed at the surface and does not penetrate into the sample. Both these effects can be minimized by the adoption of the surface optics, as is seen in Fig. 3 where the turbid and strongly absorbing (due to the high concentration of ANS) could be measured properly.

In the case of macroscopic measurement, a sample with dye concentration as low as a few micromolar can readily be measured with the same precision as in Fig. 3, where a much higher concentration was employed for comparison with the microscopic measurement.

Putting the aboves together, the nanosecond fluorometer described here is expected to be a powerful tool in the researches of biological system at cell or tissue levels.

As to the nanosecond microfluorometer, the first requirement would be the sensitivity. The very weak fluorescence from microscopic samples demands the maximal sensitivity of the detection system, which is met by the single photon counting technique.

In the ANS-ghost system, a condition of 10 ghosts in the light spot (Fig. 5) was found to be the lower limit of a meaningful observation; measurement of a single ghost gave a decay curve almost identical with that of the suspending medium. The above limit of 10 ghosts corresponds to a total of 800 μm^2 of membrane. Thus our microfluorometer is sensitive enough to allow a measurement on a single nerve fiber.[16]

The lower limit depends on one hand on the nature of the dye used. Dyes such as perylene which have greater extinction coefficient as well as greater affinity to membrane are expected to enable a decay measurement on a single ghost.

On the other hand, the light collecting efficiency of the present microfluorometer is not very high because of the relatively small numerical aperture of the reflection lenses used (0.405–0.162). The sensitivity has been sacrificed for the great working distance, which permits accurate determination of the anisotropy as well as the insertion of microelectrodes or special chambers necessary in various applications. On giving up rather the working distance, the

*S. Kawato, K. Kinoshita, Jr. and A. Ikegami: unpublished experiments.

adoption of an oil-immersion lense would increase the sensitivity by an order of magnitude.

With regard to the measuring time, it took one to two hours to obtain data comparable to Fig. 5 at a repetition rate of 5×10^3 pulses/sec. When shorter time is essential, as in the study of transient phenomena, the overall sensitivity must be increased either by observing a larger portion of the sample, choosing a more efficient dye, using an oil-immersion lense, or a combination of these.

To summarize, the nanosecond microfluorometer reported here is useful for a precise measurement of fluorescence (anisotropy) decay on relatively large samples such as nerve fibers or muscles. In case where a single cell of diameter several microns or less must be examined, selection of a suitable dye as well as some improvement of optics would be required. Use of a pulsed dye laser as an excitation source may be helpful in this regard.

As to the calculation of decay time constants, several methods have been suggested,[18] of which the method of moment[18] has been the commonest choice. With this as well as most other methods, however, φ's can be determined only indirectly. Moreover, either the peak or the tail portion of a decay time course is weighted unreasonably high. Therefore, the curve-fitting procedure described here should be preferred in the studies of the anisotropy decay, especially when both fast and slow phases are important. The program of Fletcher and Powell[12] was found to secure the efficiency of calculation.

Acknowledgment

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