shows that they are indistinguishable at all times (Figure 4.6). On a logarithmic scale, one notices some minor differences at 30–50 ns. However, at 50 ns there are only 3–5 photons per time increment (channel), so that the difference between the two decays is just 1–2 photons. In fact, if one adds the Poisson noise that is inevitably present in photon counting data, the difference between the curves is sevenfold less than the uncertainties due to the Poisson noise.[4] This illustrates that it is difficult to distinguish between some multiexponential functions or, conversely, that it is difficult to recover the actual values of α_i and τ_i for a multiexponential decay. We note that a similar result is obtained from simulations of the FD data. The simulated frequency responses are visually indistinguishable for these two decay laws.

Why is it difficult to resolve multiexponential decays? Comparison of $I_1(t)$ and $I_2(t)$ indicates that the lifetimes and amplitudes are different in each decay law. In fact, this is the problem. For a multiexponential decay, one can vary the lifetime to compensate for the amplitude, or vice versa, and obtain similar intensity decays with different values of α_i and τ_i. In mathematical terms, the values of α_i and τ_i are said to be correlated. The problem of correlated parameters has been described within the framework of general least-squares fitting.[5–7] The unfortunate result is that the ability to determine the precise values of α_i and τ_i is greatly hindered by parameter correlation. There is no way to avoid this problem, except by careful experimentation and conservative interpretation of data.

4.3. TIME-CORRELATED SINGLE-PHOTON COUNTING

At present almost all time-domain measurements are performed using TCSPC. Several comprehensive monographs dealing with TCSPC have appeared.[4,8–10] One book is completely devoted to TCSPC and provides numerous valuable details.[8] While somewhat dated, the insightful monograph of Ware[10] clearly describes the concept of TCSPC, and Ware anticipated many of its present applications. Rather than present a history of the method, we will start with current state-of-the-art instrumentation. These instruments use high-repetition-rate picosecond or femtosecond laser light sources and high-speed MCP PMTs. In later sections we will describe other light sources and detectors.

4.3.A. Principles of TCSPC

The principles of TCSPC can be understood by examination of an instrument schematic (Figure 4.7). The experiment starts with the excitation pulse, which excites the sample and starts the time measurement clock. TCSPC is a digital technique, counting photons which are time-correlated in relation to the excitation pulse. The heart of the method is a time-to-amplitude converter (TAC), which can be considered to be analogous to a fast stopwatch.

The sample is repetitively excited using a pulse light source, often from a laser or flashlamp. Each pulse is optically monitored, by a high-speed photodiode or photomultiplier, to produce a start signal which is used to trigger the voltage ramp of the TAC. The voltage ramp is stopped when the first fluorescence photon from the sample is detected. The TAC provides an output pulse whose voltage is proportional to the time between the start and stop signals. A multichannel analyzer (MCA) converts this voltage to a time channel using an analog-to-digital converter (ADC). Summing over many pulses, the MCA builds up a probability histogram of counts versus time channels. The experiment is continued until one has collected more than 10,000 counts in the peak channel. As will be described below in more detail, there can be no more than one photon detected per 100 laser pulses. Under these conditions, the histogram of photon arrival times represents the intensity decay of the sample.

There are many subtleties in TCSPC which are not obvious at first examination. Why is the photon counting rate limited to one photon per 100 laser pulses? Present electronics for TCSPC only allow detection of the first arriving photon. Once the first photon is detected, the dead time in the electronics prevents detection of another photon resulting from the same excitation pulse. Recall that emission is a random event. Following the excitation pulse, more photons are emitted at early times than at late times. If all could be measured, then the histogram of arrival times would represent the intensity decay. However, if many arrive, and only the first is counted, then the intensity decay is distorted to shorter times. This effect is described in more detail in Section 4.5.F.

Another important feature of TCSPC is the use of the rising edge of the photoelectron pulse for timing. This allows phototubes with nanosecond pulse widths to provide subnanosecond resolution. This is possible because the rising edge of the single photon pulses are usually steeper than one would expect from the time response of the PMT. Also, the use of a constant fraction discriminator provides improved time resolution by removing the variability due to the amplitude of each pulse.

4.3.B. Example of TCSPC Data

Prior to examining the electronic components in more detail, it is valuable to examine the actual data. An intensity

10 Fluorescence Anisotropy

Upon excitation with polarized light, the emission from many samples is also polarized. The extent of polarization of the emission is described in terms of the anisotropy (r). Samples exhibiting nonzero anisotropies are said to display polarized emission. The origin of these phenomena is based on the existence of transition moments for absorption and emission which lie along specific directions within the fluorophore structure. In homogeneous solution the ground-state fluorophores are all randomly oriented. When exposed to polarized light, those fluorophores which have their absorption transition moments oriented along the electric vector of the incident light are preferentially excited. Hence, the excited-state population is not randomly oriented. Instead, there is a somewhat larger number of excited molecules having their transition moments oriented along the electric vector of the polarized exciting light.

Depolarization of the emission can be caused by a number of phenomena, the relative importance of which depends upon the sample under investigation. Rotational diffusion of fluorophores is one common cause of depolarization. The anisotropy measurements reveal the average angular displacement of the fluorophore that occurs between absorption and subsequent emission of a photon. This angular displacement is dependent upon the rate and extent of rotational diffusion during the lifetime of the excited state. These diffusive motions, depend, in turn, upon the viscosity of the solvent and the size and shape of the rotating molecule. For fluorophores in solution, the rotational rate of the fluorophore is dependent upon the viscous drag imposed by the solvent. As a result, a change in solvent viscosity will result in a change in fluorescence anisotropy. For small fluorophores in solutions of low viscosity, the rate of rotational diffusion is typically faster than the rate of emission. Under these conditions, the emission is depolarized and the anisotropy is close to zero.

The dependence of fluorescence anisotropy upon rotational motion has resulted in numerous applications of fluorescence anisotropy measurements in biochemical research. This is because the timescale of rotational diffusion of biomolecules is comparable to the decay time of many fluorophores. For instance, a protein with a molecular weight of 25 kDa can be expected to have a rotational correlation time near 10 ns. This is comparable to the lifetime of many fluorophores when coupled to proteins. Hence, factors which alter the rotational correlation time will also alter the anisotropy. As examples, fluorescence anisotropy measurements have been used to quantify protein denaturation, protein association with other macromolecules, and the internal dynamics of proteins. In addition, the anisotropies of membrane-bound fluorophores have been used to estimate the internal viscosities of membranes and the effects of lipid composition upon the membrane phase-transition temperature.

In this chapter we describe the fundamental theory for steady-state measurements of fluorescence anisotropy and present selected biochemical applications. In the next chapter we will describe the theory and applications of time-resolved anisotropy measurements.

10.1. DEFINITION OF FLUORESCENCE ANISOTROPY

The measurement of fluorescence anisotropy is illustrated in Figure 10.1. The sample is excited with vertically polarized light. The electric vector of the excitation light is oriented parallel to the vertical or z-axis. One then measures the intensity of the emission through a polarizer. When the emission polarizer is oriented parallel ($\parallel$) to the direction of the polarized excitation, the observed intensity is called $I_{\parallel}$. Likewise, when the polarizer is perpendicular ($\perp$) to the excitation, the intensity is called $I_{\perp}$. These values are used to calculate the anisotropy[1]:

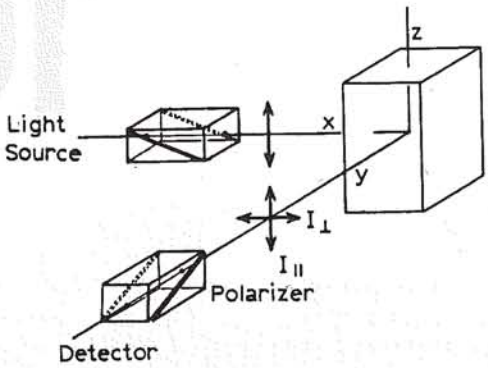

Figure 10.1. Schematic diagram for measurement of fluorescence anisotropies.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad [10.1]$$

The anisotropy is a dimensionless quantity which is independent of the total intensity of the sample. This is because the difference $(I_{\parallel} - I_{\perp})$ is normalized by the total intensity, which is $I_T = I_{\parallel} + 2I_{\perp}$.

In earlier publications one frequently encounters the term polarization, which is given by

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad [10.2]$$

The polarization and anisotropy values can be interchanged using

$$P = \frac{3r}{2 + r} \qquad [10.3]$$

$$r = \frac{2P}{3 - P} \qquad [10.4]$$

Although there is nothing incorrect about the notion of polarization, its use should be discouraged. Anisotropy is preferred because most theoretical expressions are considerably simpler when expressed in terms of this parameter, an observation first made by Alexander Jabłoński.[1] As an example of this simplification, consider a mixture of fluorophores, each with polarization P_i and a fractional fluorescence intensity f_i. The polarization of this mixture $(\overline{P})$ is given by[2]

$$\left(\frac{1}{P} - \frac{1}{3}\right)^{-1} = \sum_i \frac{f_i}{\left(\frac{1}{P_i} - \frac{1}{3}\right)} \qquad [10.5]$$

In contrast, the average anisotropy $(\overline{r})$ is given by

$$\overline{r} = \sum_i f_i r_i \qquad [10.6]$$

where the r_i indicate the anisotropies of the individual species. The latter expression is clearly preferable. Furthermore, following pulsed excitation, the decay of fluorescence anisotropy $[r(t)]$ of a sphere is given by

$$r(t) = r_0 e^{-t/\theta} \qquad [10.7]$$

where r_0 is the anisotropy at $t = 0$, and θ is the rotational correlation time of the sphere. The decay of polarization is not a single exponential, even for a spherical molecule.

Suppose that the light observed through the emission polarizer is completely polarized. Then $I_{\perp} = 0$, and $P = r = 1.0$. This value can be observed for scattered light from an optically dilute scatterer. Completely polarized emission is never observed for fluorescence from homogeneous unoriented samples. The measured values of P or r are smaller due to the angular dependence of photoselection (Section 10.2). Completely polarized emission can be observed for oriented samples.

Now suppose that the emission is completely depolarized. In this case, $I_{\parallel} = I_{\perp}$ and $P = r = 0$. However, it is important to note that P and r are not equal for intermediate values. For the moment, we have assumed that these intensities could be measured without artifacts due to the polarizing properties of the optical components, especially the emission monochromator (Section 2.3.B). In Section 10.4 we will describe methods to correct for such interference.

10.1.A. Origin of the Definitions of Polarization and Anisotropy

One may wonder why two widely used measures exist for the same phenomenon. Both P and r have a rational origin. Consider partially polarized light traveling along the x-axis (Figure 10.2), and assume that one measures the intensities I_z and I_y with the detector and polarizer positioned on the x-axis. The polarization of this light is defined as the fraction of the light that is linearly polarized. Specifically,

$$P = \frac{p}{p + n} \qquad [10.8]$$

where p is the intensity of the polarized component, and n is the intensity of the natural component. The intensity of the natural component is given by $n = 2I_y$. The remaining intensity is the polarized component, which is given by $p = I_z - I_y$. For vertically polarized excitation, $I_z = I_{\parallel}$ and $I_y = I_{\perp}$.

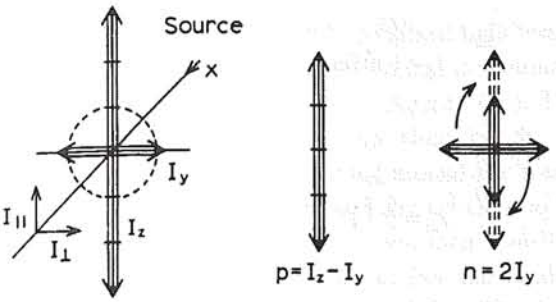

Figure 10.2. Polarization of a ray of light.

Substitution into Eq. [10.8] yields Eq. [10.2], which is the standard definition for polarization.

The anisotropy (r) of a light source is defined as the ratio of the polarized component to the total intensity (I_T),

$$r = \frac{I_z - I_y}{I_x + I_y + I_z} = \frac{I_z - I_y}{I_T} \qquad [10.9]$$

When the excitation is polarized along the z-axis, dipolar radiation from the fluorophores is also symmetric around the z-axis. Hence, $I_x = I_y$. Recalling that $I_y = I_\perp$ and $I_z = I_{||}$, one obtains Eq. [10.1].

The polarization is an appropriate parameter for describing a light source when a light ray is directed along a particular axis. In this case, $p + n$ is the total intensity, and P is the ratio of the excess intensity along the z-axis divided by the total intensity. In contrast, the radiation emitted by a fluorophore is symmetrically distributed about the z-axis.

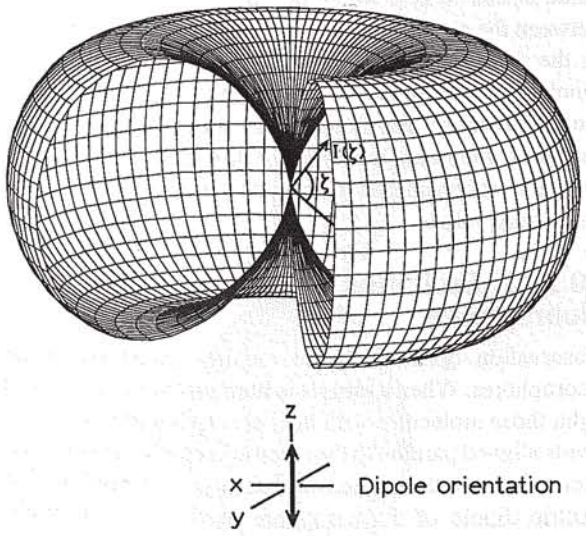

Figure 10.3. Radiating dipole in a coordinate system. The dipole is oriented along the z-axis, and the intensity $I(\zeta)$ of the emission in any direction is proportional to $\cos^2 \zeta$, where ζ is the angle from the x–y plane.

This distribution of radiated intensity is shown in Figure 10.3 for a dipole oriented along the z-axis. The intensity of the radiated light is proportional to $\cos^2 \zeta$, where ζ is the angle above or below the x–y plane. It is for this reason that, for excitation polarized along the z-axis, the total intensity is not given by $I_{||} + I_\perp$, but rather by $I_T = I_{||} + 2\,I_\perp$ (Section 10.4.F). Hence, the anisotropy is the ratio of the excess intensity that is parallel to the z-axis to the total intensity. It is interesting to notice that a dipole oriented along the z-axis does not radiate along this axis and cannot be observed with a detector on the z-axis.

10.2. THEORY FOR ANISOTROPY

The theory for fluorescence anisotropy can be derived by consideration of a single molecule.[3] Assume for the moment that the absorption and emission transition moments are parallel. This is nearly true for the membrane probe DPH. Assume that this single molecule is oriented with angles θ relative to the z-axis and ϕ relative to the y-axis (Figure 10.4). Of course, the ground-state DPH molecules will be randomly oriented in an isotropic solvent. Our goal is to calculate the anisotropy that would be observed for this oriented molecule in the absence of rotational diffusion. The conditions of parallel dipoles, immobility, and random ground-state orientation simplify the derivation.

It is known that fluorescing fluorophores behave like radiating dipoles.[4] The intensity of light radiated from a

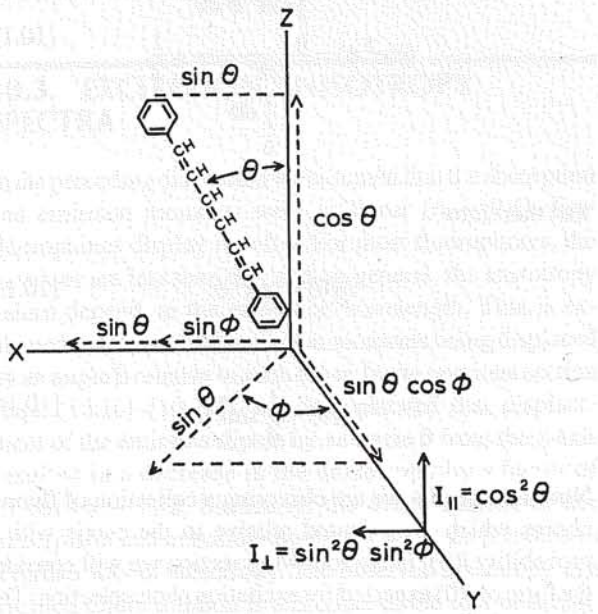

Figure 10.4. Emission intensities for a single fluorophore in a coordinate system.

dipole is proportional to the square of its vector projected onto the axis of observation. One can also reason that the emission is polarized along the transition moment. The intensity observed through a polarizer is proportional to the square of the projection of the electric field of the radiating dipole onto the transmission axis of the polarizer. These projections are given by

$$I_{\parallel}(\theta, \phi) = \cos^2 \theta \qquad [10.10]$$

$$I_{\perp}(\theta, \phi) = \sin^2 \theta \sin^2 \phi \qquad [10.11]$$

In an actual experiment the solution will contain many fluorophores with a random distribution. The anisotropy is calculated by performing the appropriate average based on excitation photoselection and how the selected molecules contribute to the measured intensity. First, consider excitation polarized along the z-axis. Such excitation must excite all molecules having an angle ϕ with respect to the y-axis with equal probability. That is, the population of excited fluorophores will be symmetrically distributed around the z-axis. Any experimentally accessible population of molecules will be oriented with values of ϕ from 0 to 2π with equal probability. Hence, we can eliminate the ϕ dependence in Eq. [10.11]. The average value of $\sin^2 \phi$ is given by

$$\langle \sin^2 \phi \rangle = \frac{\int_0^{2\pi} \sin^2 \phi \, d\phi}{\int_0^{2\pi} d\phi} = \frac{1}{2} \qquad [10.12]$$

and therefore

$$I_{\parallel}(\theta) = \cos^2 \theta \qquad [10.13]$$

$$I_{\perp}(\theta) = \frac{1}{2} \sin^2 \theta \qquad [10.14]$$

Now assume that we are observing a collection of fluorophores which are oriented relative to the z-axis with a probability $f(\theta)$. In the following section we will consider the form of $f(\theta)$ expected for excitation photoselection. The measured fluorescence intensities for this collection of molecules are

$$I_{\parallel} = \int_0^{\pi/2} f(\theta) \cos^2 \theta \, d\theta = k \langle \cos^2 \theta \rangle \qquad [10.15]$$

$$I_{\perp} = \frac{1}{2} \int_0^{\pi/2} f(\theta) \sin^2 \theta \, d\theta \, \frac{k}{2} \langle \sin^2 \theta \rangle \qquad [10.16]$$

where $f(\theta)\, d\theta$ is the probability that a fluorophore is oriented between θ and $\theta + d\theta$, and k is an instrumental constant. Using Eq. [10.11] and the identity $\sin^2 \theta = 1 - \cos^2 \theta$, one finds that

$$r = \frac{3\langle \cos^2 \theta \rangle - 1}{2} \qquad [10.17]$$

Hence, the anisotropy is determined by the average value of $\cos^2 \theta$, where θ is the angle of the emission dipole relative to the z-axis. This is because the observed intensities $I_{\parallel}$ and $I_{\perp}$ are proportional to the square of the projection of the individual transition moments onto the x- and the z-axis (Figure 10.4).

It is instructive to consider the relationship between r and θ. For a single fluorophore oriented along the z-axis, with collinear transitions, $\theta = 0$ and $r = 1.0$. However, it is not possible to obtain a perfectly oriented excited-state population with optical excitation of homogeneous solutions. Hence, the anisotropies are always less than 1.0. Complete loss of anisotropy is equivalent to $\theta = 54.7°$. This does not mean that each fluorophore is oriented at 54.7° or has rotated through 54.7°. Rather, it means that the average value of $\cos^2 \theta$ is $\frac{1}{3}$, where θ is the angular displacement between the excitation and emission moments. Recall that in the derivation of Eq. [10.17] we assumed that these dipoles were collinear. A slightly more complex expression is necessary for almost all fluorophores because the transition moments are rarely collinear. In addition, we have not yet considered the effects of photoselection on the anisotropy values.

10.2.A. Excitation Photoselection of Fluorophores

Observation of fluorescence requires excitation of the fluorophores. When a sample is illuminated with polarized light, those molecules with their absorption transition moments aligned parallel to the electric vector of the polarized excitation have the highest probability of absorption. The electric dipole of a fluorophore need not be precisely aligned with the z-axis to absorb light polarized along this axis. The probability of absorption is proportional to $\cos^2 \theta$, where θ is the angle the absorption dipole makes with

the z-axis.[3] Hence, excitation with polarized light results in a population of excited fluorophores that is symmetrically distributed around the z-axis (Figure 10.5). This phenomenon is called photoselection. Note that the excited-state population is symmetrical around the z-axis. Most of the excited fluorophores are aligned close to the z-axis, and very few fluorophores have their transition moments oriented in the x–y plane. For the random ground-state distribution, which must exist in a disordered solution, the number of molecules at an angle between θ and $\theta + d\theta$ is proportional to $\sin\theta\, d\theta$. This quantity is proportional to the surface area on a sphere within the angles θ and $\theta + d\theta$. Hence, the distribution of molecules excited by vertically polarized light is given by

$$f(\theta)\, d\theta = \cos^2\theta \sin\theta\, d\theta \qquad [10.18]$$

The probability distribution given by Eq. [10.18] determines the maximum photoselection that can be obtained using one-photon excitation of an isotropic solution. More highly oriented populations can be obtained using multiphoton excitation.[5] Recall that the anisotropy is a simple function of $\langle\cos^2\theta\rangle$ (Eq. [10.17]), so calculation of $\langle\cos^2\theta\rangle$ allows calculation of the anisotropy.

For collinear absorption and emission dipoles, the maximum value of $\langle\cos^2\theta\rangle$ is given by

$$\langle\cos^2\theta\rangle = \frac{\int_0^{\pi/2} \cos^2\theta\, f(\theta)\, d\theta}{\int_0^{\pi/2} f(\theta)\, d\theta} \qquad [10.19]$$

Substitution of Eq. [10.18] into Eq. [10.19] yields $\langle\cos^2\theta\rangle = \frac{3}{5}$. Recalling Eq. [10.17], one finds a maximum anisotropy of 0.4. This is the value which is observed when the absorption and emission dipoles are collinear, and when there are no processes which result in depolarization. Under these conditions, the excited-state population is preferentially oriented along the z-axis (Figure 10.5), and the value of $I_\perp$ is one-third the value of $I_\parallel$ ($I_\parallel = 3I_\perp$). We note that this value ($r = 0.4$) is considerably smaller than that possible for a single fluorophore oriented along the z-axis ($r = 1.0$).

It is important to remember that there are other possible origins for polarized light. These include reflections and light scattered by the sample. For a dilute scattering solution, the anisotropy is close to 1.0. Scattered light can interfere with anisotropy measurements. If the measured anisotropy for a randomly oriented sample is greater than 0.4, one can confidently infer the presence of scattered light in addition to fluorescence. The maximum anisotropy of 0.4 for collinear absorption and emission dipoles is a consequence of the $\cos^2\theta$ probability of light absorption. Anisotropy values can exceed 0.4 for multiphoton excitation (Section 10.13).

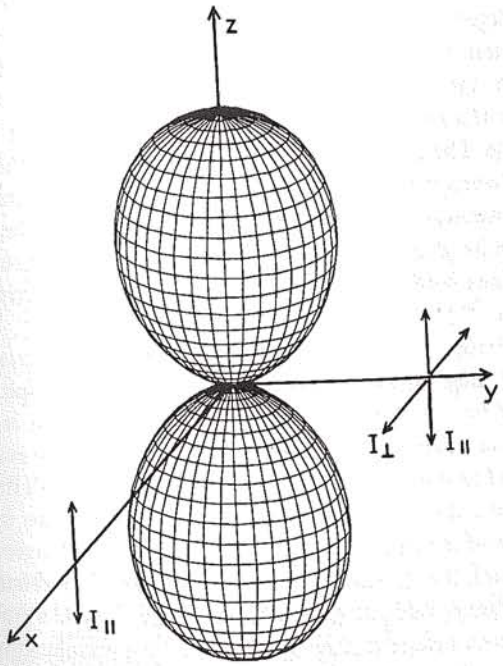

Figure 10.5. Excited-state distribution for immobile fluorophores with $r_0 = 0.4$.

10.3. EXCITATION ANISOTROPY SPECTRA

In the preceding discussion we assumed that the absorption and emission moments were collinear ($r_0 = 0.4$). Few fluorophores display $r_0 = 0.4$. For most fluorophores, the r_0 values are less than 0.4, and, in general, the anisotropy values depend on the excitation wavelength. This is explained in terms of the transition moments being displaced by an angle β relative to each other. In the previous section (Eqs. [10.10]–[10.17]), we demonstrated that displacement of the emission dipole by an angle θ from the z-axis resulted in a decrease in the anisotropy by a factor of $(3\cos^2\theta - 1)/2$. Similarly, the displacement of the absorption and emission dipoles by an angle β results in a further loss of anisotropy. The observed anisotropy in a vitrified dilute solution is a product of the loss of anisotropy due to photoselection (resulting in a reduction of the anisotropy by a factor of $\frac{2}{5}$) and that due to the angular

11

Time-Dependent Anisotropy Decays

In the preceding chapter we described the measurement and interpretation of steady-state fluorescence anisotropies. These values are measured using continuous illumination and represent an average of the anisotropy decay over the intensity decay. Measurement of steady-state anisotropies is simple, but interpretation of the steady-state anisotropies usually depends on an assumed form for the anisotropy decay, which is not directly observed in the experiment. Additional information is available if one measures the time-dependent anisotropy, that is, the values of $r(t)$ following pulsed excitation. The form of the anisotropy decay depends on the size, shape, and flexibility of the labeled molecule, and the data can be compared with the decays calculated from various molecular models. Anisotropy decays can be obtained using the TD or the FD method.

It is valuable to understand the factors which affect the anisotropy decays. For a spherical molecule, the anisotropy is expected to decay with a single rotational correlation time (θ). Perhaps the most frequent interpretation of the correlation time is in terms of the overall rotational correlation time of a protein. The measured values of θ can compare with the value predicted for a hydrated sphere of equivalent molecular weight (Eq. [10.52]). However, numerous factors can result in multiexponential anisotropy decays. Multiexponential anisotropy decays are expected for nonspherical fluorophores or proteins. In this case the correlation times are determined by the rates of rotation about the various molecular axes. Anisotropy decays can be used to estimate the shapes of proteins.

In addition to being affected by shape, anisotropy decays are affected by the segmental flexibility of the macromolecule. For instance, tryptophan anisotropy decays of proteins frequently display correlation times that are too short to be due to rotational diffusion of the whole macromolecule. These short-correlation-time components are often due to independent motions of the tryptophan residue within the protein. Measurements of these components have been widely used to understand the internal dynamics of proteins. Anisotropy decays can also be affected by RET between molecules of the same type of fluorophore, that is, depolarization due to homotransfer.

Anisotropy decays of membrane-bound probes have been particularly informative. Membrane-bound probes often display unusual behavior, whereby the anisotropies do not decay to zero. This behavior occurs because, in contrast to probes in isotropic solutions, probes in membranes do not rotate freely. The extent of rotation is often limited by the anisotropic environment of a membrane. The nonzero anisotropies at long times have been interpreted in terms of the order parameters of the membrane. In this chapter we present examples of simple and complex anisotropy decays to illustrate the wealth of information available from measurements of time-dependent anisotropies. In the following chapter we describe more advanced concepts in anisotropy decay analysis.

11.1. ANALYSIS OF TIME-DOMAIN ANISOTROPY DECAYS

Suppose that a fluorophore is excited with a pulse of vertically polarized light and that it rotates with a single correlation time. The anisotropy decay is determined by measuring the decay of the vertically (||) and horizontally ($\perp$) polarized components of the emission. If the absorption and emission transition moments are collinear, the time-zero anisotropy is 0.4. In this case the initial intensity of the parallel component is threefold larger than that of the perpendicular component (Figure 11.1, left). Assuming that the fundamental anisotropy is greater than zero ($r_0 > 0$), the vertically polarized excitation pulse results in an initial population of fluorophores that is enriched in the parallel orientation. The decay of the difference between $I_{\parallel}(t)$ and $I_{\perp}(t)$, when properly normalized by the total intensity, is the anisotropy decay (Figure 11.1, right).

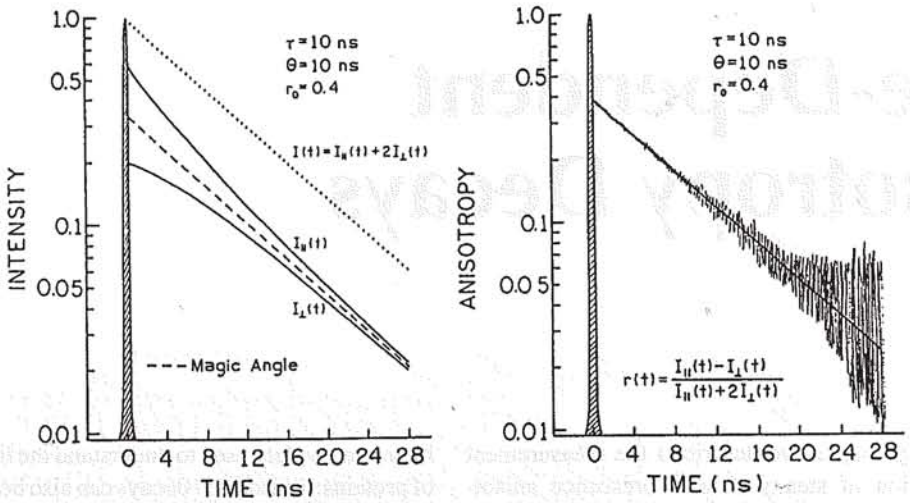

Figure 11.1. Time-dependent polarized decays (*left*) and the calculated anisotropy decay (*right*). From Ref. 1.

Examination of the left panel in Figure 11.1 reveals that the parallel component initially decays more rapidly than the horizontal component. This occurs because the vertically oriented fluorophores are decaying by two processes, the intensity decay with decay time τ and rotation out of the vertical orientation with a correlation time θ. The horizontal component initially decays more slowly because it is repopulated by rotation from the excess vertically oriented population.

Interpretation of anisotropy decays is best understood in terms of the individual components. The decays of the parallel ($\|$) and perpendicular ($\perp$) components of the emission are given by

$$I_{\|}(t) = \frac{1}{3} I(t)[1 + 2r(t)] \qquad [11.1]$$

$$I_{\perp}(t) = \frac{1}{3} I(t)[1 - r(t)] \qquad [11.2]$$

where $r(t)$ is the time-resolved anisotropy. Generally, $r(t)$ can be described as a multiexponential decay,

$$r(t) = r_0 \sum_j g_j \exp(-t/\theta_j) = \sum_j r_{0j} \exp(-t/\theta_j) \qquad [11.3]$$

where $r_0 = \Sigma_j r_{0j}$ is the limiting anisotropy in the absence of rotational diffusion, the θ_j are the individual correlation times, and the g_j are the associated fractional amplitudes in the anisotropy decay ($\Sigma g_j = 1.0$). Depending on the circumstances, r_0 may be a known parameter, perhaps from a frozen solution measurement. Alternatively, all the amplitudes (r_{0j}) can be considered to be experimental variables. As shown in the previous chapter, the total intensity for a sample is given by $I_T = I_{\|} + 2I_{\perp}$. Similarly, the total (rotation-free) intensity decay is given by

$$I(t) = I_{\|}(t) + 2I_{\perp}(t) \qquad [11.4]$$

In the time domain, one measures the time-dependent decays of the polarized components of the emission (Eqs. [11.1] and [11.2]). The polarized intensity decays are used to calculate the time-dependent anisotropy,

$$r(t) = \frac{I_{\|}(t) - I_{\perp}(t)}{I_{\|}(t) + 2I_{\perp}(t)} \qquad [11.5]$$

The time-dependent anisotropy decay is then analyzed to determine which model is most consistent with the data.

The experimental procedures and the form of the data are different for FD measurements of the anisotropy decays.[2] The sample is excited with amplitude-modulated light which is vertically polarized (Figure 11.2). As for the TD measurements, the emission is observed through a polarizer, which is rotated between the parallel and the perpendicular orientations. In the frequency domain, there are two observable quantities which characterize the anisotropy decay. These are the phase shift Δ_ω, at the modulation frequency ω, between the perpendicular ($\phi_\perp$) and parallel ($\phi_\|$) components of the emission,

$$\Delta_\omega = \phi_\perp - \phi_\| \qquad [11.6]$$

and the ratio of the parallel ($m_\|$) and the perpendicular ($m_\perp$) components of the modulated emission,