Fluorescence Polarization/Anisotropy in Diagnostics and Imaging

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Received August 4, 2009

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1. Introduction

Fluorescence polarization was first applied in biochemistry almost 6 decades ago, when Gregorio Weber described his studies on bovine serum albumin and ovalbumin conjugated with 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride)[1,2] (Figure 1). (For an overview of Gregorio Weber's wide-ranging contributions to fluorescence, see ref 3). Polarization methods became increasingly popular during the decades following Weber's work. During the past few decades, however, the increase in the number and diversity of fluorescence polarization studies has been astonishing and the method is now extremely widespread in the clinical and biomedical fields. The virtual explosion of polarization studies, which began during the mid-1980s, was due to several factors, including the availability of commercial instruments equipped with polarizers, the commercial availability of a great many fluorescence probes (largely due to the company Molecular Probes—now part of Invitrogen), and, in the clinical chemistry area, the introduction of the TDx instrument (and associated reagents) by Abbott Laboratories. The reasons for the popularity of fluorescence polarization in clinical and high-throughput assays are manifold. First, polarization assays are homogeneous; that is, there is no necessity for separation of free and bound ligand (these types of assays are often referred to as "mix and measure" assays). Second, and one of the original motivations for the development of fluorescence-based assays, there is no need for radioisotopes. Third, polarization assays are reproducible and facilely automated. In this review we shall briefly trace the history of the technique and discuss in detail both theoretical and practical aspects. We shall also present copious examples from the literature, which illustrate the scope of the method in areas such as ligand binding, immunoassays, high-throughput screening, and live cell imaging and hint at future directions and applications.

2. Historical Overview

As mentioned above, fluorescence polarization is widely applied in diverse fields, especially in the life sciences. A rough survey using Pubmed reveals that publications on

10.1021/cr900267p
Published on Web 03/16/2010

David M. Jameson earned his Ph.D. under the supervision of Gregorio Weber at the University of Illinois at Urbana−Champaign. His main research interests are the application of fluorescence methodologies, including time-resolved fluorescence and fluorescence microscopy, to biophysical and biochemical problems, both in vitro and in living cells. He is particularly interested in protein−protein and protein−ligand interactions. His research has been funded by the National Science Foundation, the American Heart Association, and the National Institutes of Health. He is presently a Professor in the Department of Cell and Molecular Biology at the University of Hawaii. Every three years he co-organizes the International Weber Symposium on Innovative Fluorescence Methodologies in Biochemistry and Medicine held in Hawaii.

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fluorescence polarization/anisotropy numbered around a dozen in the 1950s, less than 100 in the 1960s, several hundred in the 1970s, more than two thousand in the 1980s, more than three thousand in the 1990s, and close to four thousand in the 2000s to date. Given this rising trend, one may be curious how it all got started. We shall thus present a brief history of fluorescence polarization.

2.1. Discovery of Polarization

In 1808, Etienne-Louis Malus observed sunlight reflected from the windows of the Luxemburg Palace in Paris through an Iceland spar (Calcite) crystal that he rotated (Erasmus Bartholin had discovered the double refraction of light by an Iceland spar in 1669). Malus discovered that the intensity of the reflected light varied as he rotated the crystal and coined the term "*polarized*" to describe this property of light. He published his findings in 1809.[4] Malus also derived an

Figure 1. Gregorio Weber in Hawaii in 1989. Photograph courtesy of Prof. David Jameson.

expression for calculating the transmission of light as a function of the angle (θ) between two polarizers. This equation (Malus' Law) is now written as $I(\theta) = I_0(\cos^2 \theta)$. Some years later, David Brewster studied the relationship between refractive index and angle of incidence on the polarization of the reflected light.[5] He discovered that for normal glass and visible light, an incidence angle of ~56° resulted in total reflection of one plane of polarization—this angle is now known as Brewster's Angle. This discovery allowed Brewster to construct a polarizer composed of a "pile of plates" (interestingly, when one of the authors—D.M.J.—first joined Gregorio Weber's laboratory as a graduate student, Weber showed him the "pile of plates" polarizer that he had used in some of his initial studies). In 1828, William Nicol joined two crystals of Iceland spar, cut at an angle of 68°, using Canada balsam, which allowed a spatial separation between the orthogonally polarized components. Other important calcite polarizers developed around this time include the following: Glan−Foucault; Glan−Thompson; Glan−Taylor; Wollaston; and Rochon. Most modern spectrofluorimeters today use Glan−Taylor type calcite polarizers, which have an air-gap between the two calcite crystals allowing for transmission deep into the ultraviolet and which makes them less susceptible to photodamage at higher irradiance levels. But the Henry Ford of polarizers was Edwin Herbert Land. In 1929 Edwin Land patented the sheet polarizer (the J-sheet), consisting of crystals of iodoquinine sulfate embedded in nitrocellulose film followed by alignment of the crystals by stretching, which led to dichroism. In 1937 he founded the Polaroid Company, and in 1938 he invented the H-sheet, which comprised polyvinyl alcohol sheets with embedded iodine. Land's invention made him quite rich and also allowed the use of inexpensive polarizers in diverse applications such as sunglasses and photography filters.

2.2. Fluorescence Polarization

Although the phenomenon now known as "fluorescence" was reported as early as the mid-1500s by the Aztecs (see, for example, the recent excellent treatises by Acuña on the

XXX. *On the Change of Refrangibility of Light.* *By* G. G. Stokes, *M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge.*

Received May 11,—Read May 27, 1852.

1. THE following researches originated in a consideration of the very remarkable phenomenon discovered by Sir John Herschel in a solution of sulphate of quinine, and described by him in two papers printed in the Philosophical Transactions for 1845, entitled 'On a Case of Superficial Colour presented by a Homogeneous Liquid internally colourless,' and 'On the Epipolic Dispersion of Light.' The solution of quinine, though it appears to be perfectly transparent and colourless, like water, when viewed by transmitted light, exhibits nevertheless in certain aspects, and under certain incidences of the light, a beautiful celestial blue colour. It appears from the experiments of Sir John Herschel that the blue colour comes only from a stratum of fluid of small but finite thickness adjacent to the surface by which the light enters. After passing through this stratum, the incident light, though not sensibly enfeebled nor coloured, has lost the power of producing the same effect, and therefore may be considered as in some way or other qualitatively different from the original light. The dispersion which takes place near the surface of this liquid is called by Sir John Herschel *epipolic*, and he applies the term *epipolized* to a beam of light which, having been transmitted through a quiniferous solution, has been thereby rendered incapable of further undergoing epipolic dispersion. In one experiment, in which sun-light was used, a feeble blue gleam was observed to extend to nearly half an inch from the surface. As regards the dispersed light itself, when analysed by a prism it was found to consist of rays extending over a great range of refrangibility: the less refrangible extremity of the spectrum was however wanting. On being analysed by a tourmaline, it showed no signs of polarization. A special experiment showed that the

Figure 2. Photo of George Gabriel Stokes (retrieved on July 26, 2009 from http://commons.wikimedia.org/wiki/File:Stokes_George_G.jpg) and the first page of his famous manuscript. Reprinted with permission from ref 8. Copyright 1852 The Royal Society.

early observations on *Lignum nephriticum*[6,7]), the essential realization that the phenomenon was due to light absorption followed by emission was first enunciated by George Gabriel Stokes.

2.2.1. Stokes

The history of fluorescence polarization can be traced to Stokes' famous 1852 manuscript[8] (Figure 2), "On the Change of Refrangibility of Light", where he actually coined the word "fluorescence" to replace the term "dispersive reflection" in a footnote stating "I confess I do not like this term. I am almost inclined to coin a word, and call the appearance *fluorescence* from fluor-spar as the analogous term *opalescence* is derived from the name of a mineral". During his studies, Stokes used a prism to disperse the solar spectrum and illuminate a solution of quinine. He noted that there was no effect until the solution was placed in the ultraviolet region of the spectrum. He wrote: "It was certainly a curious sight to see the tube instantaneously lighted up when plunged into the invisible rays: it was literally *darkness visible*. Altogether the phenomenon had something of an unearthly appearance." This observation led Stokes to proclaim that fluorescence is of longer wavelength than the exciting light, which led to this displacement being called the Stokes shift. Stokes actually isolated the emission of quinine from the exciting light using colored filters, some of which he obtained from his friend, Michael Faraday, and viewed this emission through Nicol polarizers. He reported that there was no apparent polarization, which actually was correct, since the lifetime of quinine is ~20 ns, sufficiently long such that reorientation of the excited fluorophore is virtually complete during its excited state lifetime (the relationship between polarization and lifetime will be discussed in detail later in this review).

2.2.2. Weigert

It was not until 1920 that polarized fluorescence was observed from aqueous solutions of small dye molecules by Weigert.[9] Weigert wrote: "Der Polarisationsgrad des Fluorezenzlichtes nimmt mit wachsender Molekulargrösse, mit zunehmender Viskosität des Mediums und mit abnehmender Temperatur, also mit Verringerung der Beweglichkeit der Einzelteilchen zu", which may be translated as "The degree of the polarization increases with increasing molecular size, with increasing viscosity of the medium and with decreasing temperature, that is with the reduction of the mobility of the single particles." Hence, Weigert recognized that fluorescence polarization increased as the mobility of the emitting species decreased. The first comprehensive study of this newly discovered phenomenon was due to Vavilov and Levshin in 1923,[10] who measured the polarization of 26 dyes in water and glycerol. They were able to demonstrate that some of the dyes they studied showed large differences between polarizations in water compared to the polarizations in glycerol, whereas other dyes gave similar polarizations regardless of the solvent's viscosity (which the astute reader will realize was due to the very short fluorescence lifetimes of this second group of dyes). These prescient observers in fact postulated that the fluorescence was due to molecular rotation of a fluorophore characterized by an electric vector that could oscillate only in one direction, which lead them to correctly calculate that the maximum values of the polarization would be $^1/_2$ for such a linear oscillator and $^1/_7$ for a circular oscillator (the limits of polarization for fluorophores in solution will be discussed later).

2.2.3. Perrin and Gaviola

Francis Perrin, son of the famous physicist Jean Perrin, then published a series of definitive papers which derived

the relationships between polarization and the rotation and excited state lifetimes of fluorophores.[11−13] A very important paper in the history of fluorescence polarization and the phenomenon which would become known as FRET (Förster Resonance Energy Transfer) was published by Enrique Gaviola and Peter Pringsheim in 1924.[14] This paper reported the finding that the polarization of uranin (sodium fluorescein) in glycerol was dramatically reduced as the concentration of the dye increased. This observation was recognized to be due to transfer of energy between fluorescein molecules manifested by a loss in the orientation of the excited state and subsequent depolarization. Subsequently, the Perrins (father and son) and others proposed quantitative theories for resonance energy transfer, culminating in the work of Theodor Förster.

2.2.4. Weber

Polarization remained largely in the province of the physicists for almost two decades, until Gregorio Weber began his thesis work with the famous enzymologist Malcolm Dixon at Cambridge in the mid-1940s. In later years, Weber would say that the work that most influenced him and which he liked the best was that of Francis Perrin. Weber's introduction to polarization started when he read Perrin's classic paper of 1926 in the *Journal de Physique* on the depolarization of fluorescence by Brownian rotations.[13] In Weber's own words,[15] "I remember that Malcolm Dixon came to me one day, handed me a little piece of paper, and said that somebody at King's College—I wish I could remember his name—had said that there was a paper on fluorescence that I should read. The little piece of paper had written on it: F. Perrin, *J. de Physique*, 1926. So I went to the Cambridge library, which I positively thought of as a temple of learning and looks indeed like one, and I read the famous paper of Perrin on depolarization of the fluorescence by Brownian rotations, not one but many times. Argentine secondary education in the first half of the century included French language and literature so that I could not only understand the scientific content, but also enjoy the literary quality of the writing. It was written in that transparent, terse style of XVIII century France, which I have tried, perhaps unsuccessfully, to imitate from then onwards. The clarity of Perrin's thought and his ability to do the right experiment were really remarkable." Weber went on to note "It was from reading Perrin's papers that I conceived three ideas on the use of polarization: determination of the change in the fluorescence lifetime as one quenches the fluorescence by addition of an appropriate chemical, determination of the molecular volume of proteins by fluorescent conjugates with known dyes and determination of the viscosity of a medium through the polarization of the emission from a known fluorescent probe." Weber's subsequent theoretical and experimental work extended Perrin's earlier contributions, and he also developed what became modern instrumentation. These efforts brought fluorescence polarization to the attention of the biochemical community and so ushered in a new scientific discipline—quantitative biological fluorescence.

3. Basic Principles of Polarization

The underlying principles of fluorescence polarization, e.g., the effect of the fluorescence lifetime, excitation wavelength, molecular mass, and solvent viscosity, have been discussed many times in the literature in varying degrees of detail (e.g., refs 16−21 to list but a few). In this section we will present an overview, which will focus on what we believe to be the most important concepts for an appreciation of the application of fluorescence polarization to biological fluorescence.

3.1. Definitions: Polarization and Anisotropy

Light can be considered as oscillations of an electromagnetic field—characterized by electric and magnetic components—perpendicular to the direction of light propagation (Figure 3). For our purposes we shall only be concerned with the electric field component. In natural light the electric field vector can assume any direction of oscillation normal to the light propagation direction. Polarizers, as indicated earlier, are optically active devices that can isolate one direction of the electric vector. Consider an *XYZ* coordinate framework with a fluorescent solution placed at the origin, as shown in Figure 4A, where *XZ* is in the plane of the page. If an oscillating dipole is placed so that the line of separation of charge, the dipole axis, coincides with the Z axis, the energy radiated during the oscillations is symmetric about *Z*. The amplitude of the generated electric field in the space surrounding the dipole is proportional to cos θ, and the intensity of the irradiation is proportional to $\cos^2 \theta$. In this system, the exciting light is traveling along the *X* direction. If a polarizer is inserted in the beam, one can isolate a unique direction of the electric vector and obtain light polarized parallel to the *Z* axis, which corresponds to the vertical laboratory axis. This exciting light will be absorbed by the fluorophore at the origin and give rise to fluorescence, which is typically observed at 90° to the excitation direction, i.e., along the *Y* axis. The actual direction of the electric vector of the emission can be determined by viewing the emission through a polarizer which can be oriented alternatively in the parallel or perpendicular direction relative to the *Z* axis or laboratory vertical

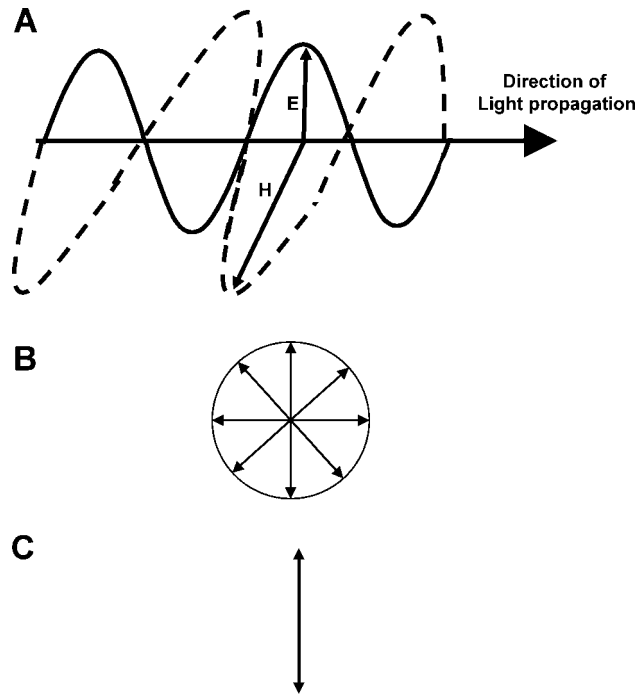

Figure 3. (A) Sketch depicting oscillations of the electric (E) and magnetic (H) fields for a propagating electromagnetic wave. Orientation of the electric field in natural/unpolarized light (B) and vertically polarized light (C).

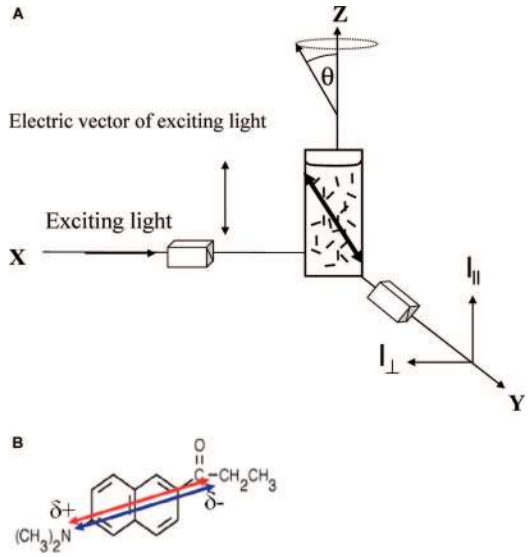

Figure 4. (A) Illustration of the relative axes of the laboratory coordinate system showing the orientation of the electric field vector of the excitation light and the relative orientations of the excitation and emission polarizer, and the angle of the rotating molecules with respect to the Z axis (θ). (B) Chemical structure of PRODAN showing the absorption (blue) and emission (red) dipole moments.

direction. Polarization is then defined as a function of the observed parallel ($I_{||}$) and perpendicular intensities ($I_{\perp}$):

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} \tag{1}$$

If the emission is completely polarized in the parallel direction, i.e., the orientation of the electric vector of the exciting light is totally maintained, then

$$P = \frac{1 - 0}{1 + 0} = 1 \tag{2}$$

If the emitted light is totally polarized in the perpendicular direction then

$$P = \frac{0 - 1}{0 + 1} = -1 \tag{3}$$

In these cases, then, the limits of polarization would be $+1$ to -1, and such limits can be reached in completely oriented systems, e.g., crystals. Another term frequently used in the context of polarized emission is anisotropy (introduced in 1960 by Jabłoński[22]), usually designated as either A or r, which is defined as

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \tag{4}$$

By analogy to polarization, the limits of anisotropy in completely oriented systems are $+1$ to -0.5. Given the definition of polarization and anisotropy, one can show that

$$r = \frac{2}{3}\left(\frac{1}{P} - \frac{1}{3}\right)^{-1} \tag{5}$$

or

$$r = \frac{2P}{3 - P} \tag{6}$$

The information content of the polarization function and the anisotropy function is identical for most purposes (see ref 23 for a detailed discussion of formal information content), and the use of one term or the other is dictated by practical considerations and custom. For example, one useful aspect of the anisotropy function is that the denominator, $I_{||} + 2I_{\perp}$, represents the total intensity of an emission excited by parallel polarized light. In clinical chemistry fields, the polarization function is used almost exclusively, whereas in biophysics and biochemistry the anisotropy function is more common.

3.2. Limiting (Intrinsic) Polarization

In solutions, the theoretical limits of ± 1 for polarization are not realized. Consider, as shown in Figure 4A, a cuvette containing a solution of fluorophores at the origin of our coordinate system. Upon absorption of an exciting photon, a dipole moment is created in the fluorophore (usually of different magnitude and direction from the ground state dipole). The orientation of this dipole moment relative to the nuclear framework, and its magnitude, will be determined by the nature of the substituents on the molecule (Figure 4B). This excited state dipole moment is also known as the transition dipole or transition moment. In fact, if light of a particular electric vector orientation (plane polarized light) impinges on a sample, only those molecules which are appropriately oriented relative to this electric vector can absorb the light. Specifically, the probability of the absorption is proportional to the cosine squared ($\cos^2 \theta$) of the angle θ between the exciting light and the transition dipole. Hence, when we excite an ensemble of randomly oriented fluorophores with plane-polarized light, we are performing a *photoselection* process, creating a population of excited molecules which nominally have their excited dipoles lined up with the polarization direction of the excitation (this photoselection process is illustrated in Figure 5).

Consider now that the transition dipole corresponding to the emission of light from the excited fluorophore is parallel to the absorption dipole (Figure 4B) and that the excited fluorophore cannot rotate during the lifetime of the excited state (for example if the fluorophores are embedded in a highly viscous or frozen medium). If we

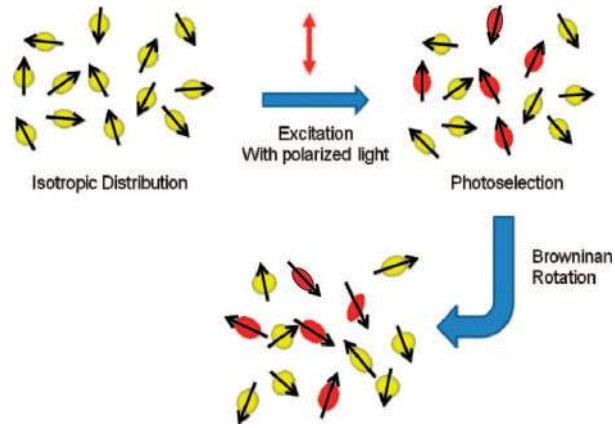

Figure 5. Illustration of the photoselection of a randomly oriented distribution of fluorophores by vertically polarized light and their subsequent rotational Brownian diffusion.

were to now measure the polarization of the emission, it would be less than +1, since some of the dipoles excited will not be exactly parallel to the direction of the exciting light. In fact, the number of potential dipoles making an angle θ with the vertical axis will be proportional to sin θ. We can then calculate that the upper polarization limit for such a randomly oriented (but rigidly fixed, i.e., nonrotating) ensemble—with colinear excitation and emission dipoles—will be $+^1/_2$. If the absorption and emission dipoles are at 90° to each other, then considering the same $\cos^2 \theta$ photoselection rule and the sin θ population distribution as before, one can show that the polarization will be equal to $-^1/_3$. For an explicit derivation of these limits, the reader may refer to the excellent treatise by Weber.[16] The limiting anisotropies, as calculated using eq 5 or 6, are +0.4 and −0.2. Consider the general case shown in Figure 6 (adapted from Weber[16]), which depicts two principle absorption bands for phenol. The energy level diagram, or Perrin−Jabłoński diagram, corresponding to this system, is also depicted. The directions of the absorption dipoles—relative to the nuclear framework—differ greatly for the two transitions as illustrated; specifically, the transitions known as ^1L_a and ^1L_b are indicated. The two excited dipoles corresponding to these transitions correspond to the $S_0 \rightarrow S_1$ and the $S_0 \rightarrow S_2$ bands sketched in the absorption spectrum. In fact, as indicated, these dipoles are oriented at a large angle with respect to one another—approximately 90°. After the excitation process, however, regardless of whether the absorption process corresponded to the $S_0 \rightarrow S_1$ or the $S_0 \rightarrow S_2$ transition, rapid thermalization leaves the excited fluorophore in the S_1 level and emission will result from the $S_1 \rightarrow S_0$ transition. If excitation corresponded to the $S_0 \rightarrow S_2$, e.g., excitation near 235 nm, the orientation of the absorption dipole will possess a different average orientation than the absorption dipoles photoselected by 280 nm excitation. Hence, with 235 nm excitation, more emission is observed in the perpendicular direction than in the parallel direction, and the resulting polarization will be negative.

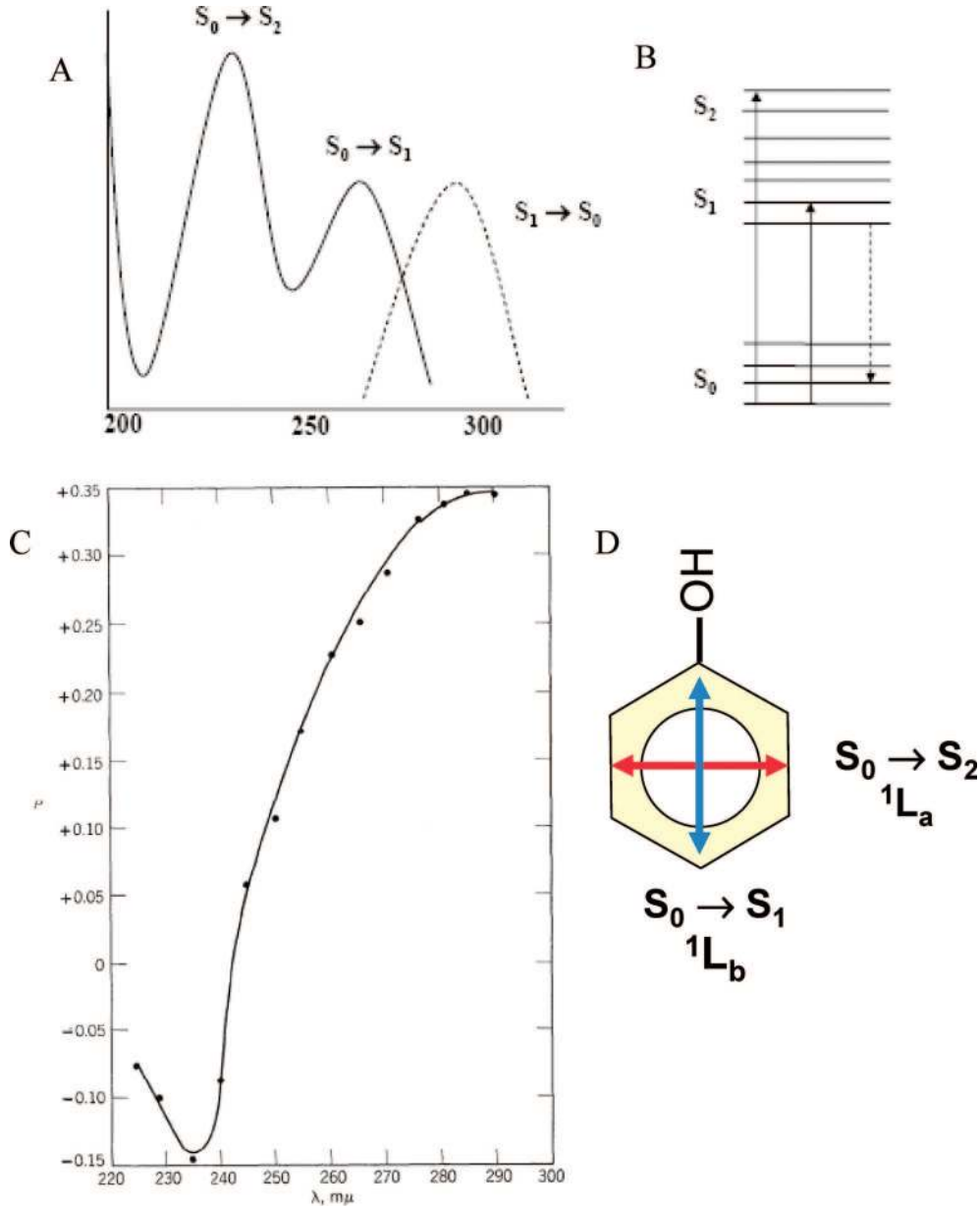

Figure 6. (A) Excitation and emission spectra, (B) Perrin−Jabłoński energy level diagram, (C) excitation polarization spectrum, and (D) chemical structure illustration of the orientations of the ^1L_b and ^1L_a ($S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$) electronic transitions of phenol. Reprinted with permission from ref 16. Copyright 1966 John Wiley & Sons Inc.

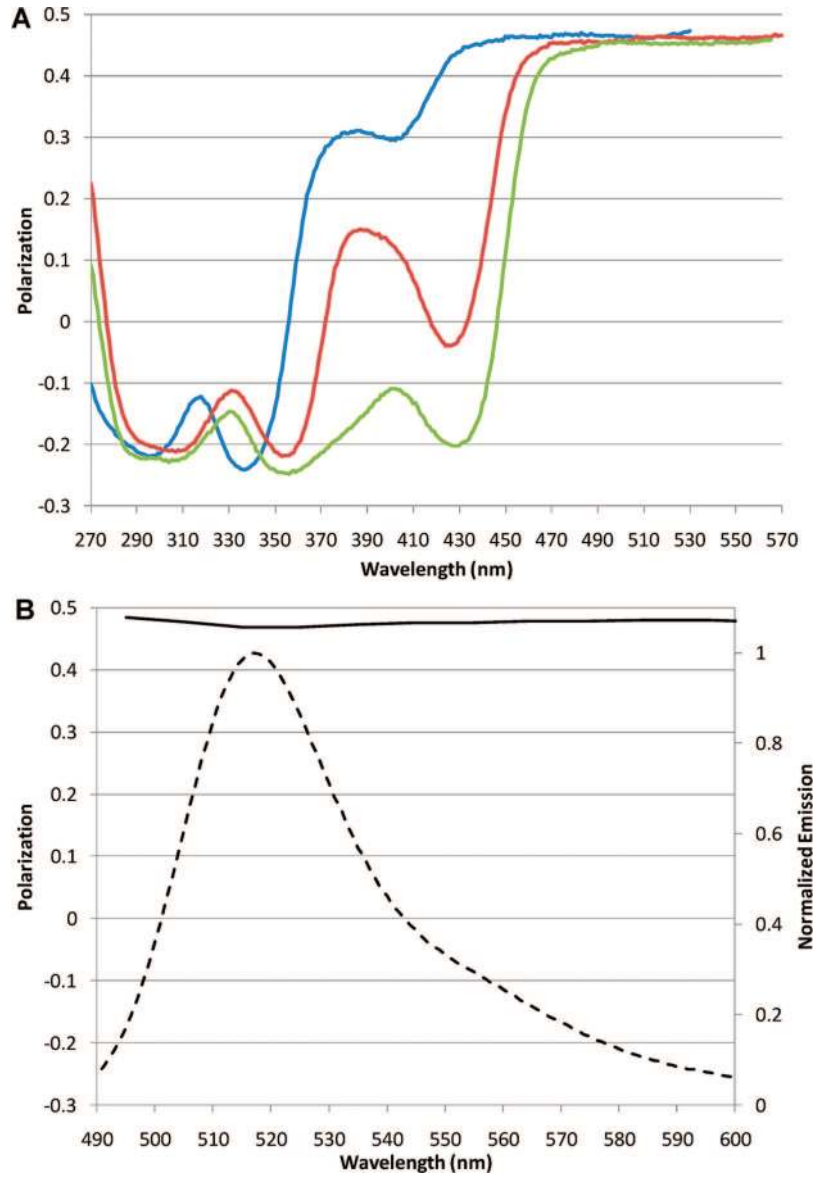

Figure 7. (A) Excitation polarization spectra of Rhodamine 123 (Rh 123, blue), Rhodamine B methyl ester (Rh B-Me, red),[186] and tetramethylrhodamine maleimide reacted with free cysteine (TMR, green) in glycerol pH 7 at 2 °C. (B) Emission intensity (dashed) and emission polarization scan (solid) of fluorescein, excited at 480 nm, in glycerol with 0.01 M NaOH at 2 °C.

These polarization values, in the absence of rotation, are termed limiting or intrinsic polarizations and are denoted as P_0. In general

$$\frac{1}{P_0} - \frac{1}{3} = \frac{5}{3}\left(\frac{2}{3\cos^2\phi - 1}\right) \quad (7)$$

where ϕ is the angle between the absorption and emission dipoles. We can thus understand that the limiting polarization of a fluorophore will depend upon the excitation wavelength. Plots showing this variation of intrinsic polarization with excitation wavelength are termed "excitation polarization spectra", and that corresponding to the phenol case just discussed is also shown in Figure 6. Several other excitation polarization spectra are shown in Figure 7A for various rhodamines (to be discussed in greater detail later), which illustrates the general property of varying orientations of absorption and emission bands. The limiting polarization is almost always independent of the emission wavelength, as shown for example for the case of fluorescein in Figure 7B. In some rare cases, however, typically when the vibronic structure of the emission is evident, it can vary with emission wavelength. For example, in the case of chrysene, the limiting polarization varies across the emission band.[21] Pyrene, a commonly used long-lifetime probe, also has intrinsic polarization properties that vary with both excitation and emission wavelengths, and hence, one must choose these wavelengths carefully to maximize the polarization.

3.3. Fluorophore Rotation and Polarization/Anisotropy

The utility of fluorescence polarization in the clinical and biomedical sciences ultimately rests on the dependence of the observed polarization on the rotational diffusion rate of molecules (in most cases—the exception being fluorescence polarization changes due to FRET, as discussed later). We shall initially approach this topic in an intuitive and qualitative manner and only then consider a quantitative treatment.

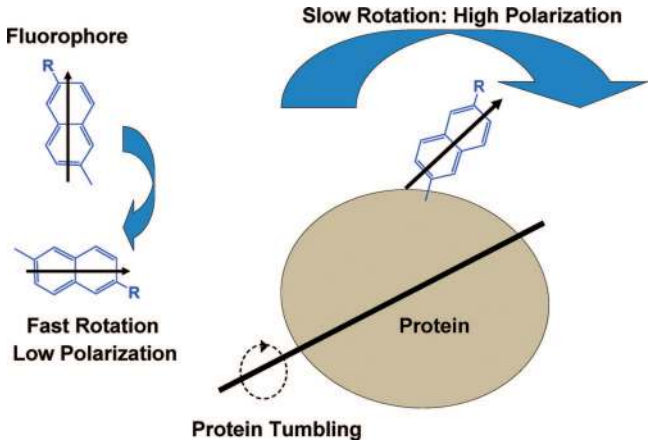

Figure 8. Illustration of the rotation of a fluorophore free in solution as compared to a fluorophore attached to a protein or macromolecule. As indicated in the figure, the fluorophore free in solution can rotate rapidly (compared to its fluorescence lifetime) and hence gives rise to a low polarization value whereas a fluorophores attached to a protein (either covalently or noncovalently) will rotate more slowly (relative to its fluorescence lifetime), due to the larger mass of the macromolecule, and hence give rise to a high polarization.

3.3.1. Qualitative Considerations

If we imagine a fluorescent molecule in a solution, we may consider what happens when a photon is absorbed. If the solute surrounding the excited fluorophore is completely rigid, then the orientation of the nuclear framework will not vary between the moment of excitation and emission. In that case, the polarization will equal the limiting or intrinsic polarization realized at that particular excitation wavelength, which reflects the orientation of the absorption and emission dipoles. If the molecule is free to rotate, however, then the observed polarization will be influenced not only by the limiting polarization but also by the extent of rotation experienced by the molecule before light is emitted (illustrated in Figure 5). Clearly, the extent of rotation will depend upon how fast the molecule can rotate before it emits a photon. We can easily imagine, as did Weigert in 1920 (section 2.2.2), that the rotational rate will be slower as the molecule gets larger. For example, the rotational rate of a small molecule, e.g., of molecular weight 300 Da, will be much faster than the rotational rate of a large molecule, e.g., 100,000 Da. During the same fixed time interval, we would thus expect the small molecule to rotate through a larger angle than the large molecule and, hence, we would expect that the light emitted by the smaller molecule will be more equally distributed in all directions, compared to the case of the larger molecule, whose emission will be oriented more or less in line with the original excitation direction. This scenario is illustrated in Figure 8. The time interval of interest, of course, is the fluorescence lifetime. Essentially, we may think of the fluorophore as a *molecular stopwatch*, which starts with the absorption of a photon and stops with the emission of a photon. We can only observe events which occur during this time interval. Hence, we can qualitatively understand the effect of molecular size and the fluorescence lifetime on the polarization. Clearly, the shorter the lifetime the less the fluorophore will rotate between the time of absorption and emission and the higher will be the polarization of the emitted light. Consider a typical small fluorescent ligand with a rotational relaxation time of ~1 ns. If the fluorescent lifetime is 0.1 ns, then the polarization observed for the free ligand will be quite high—near the limiting polarization—since the probe does not have time to rotate appreciably before emission. On the other hand, if the probe lifetime is much longer, e.g., ~10 ns, then the free fluorescent ligand can have a low polarization. In fact, before the development of instrumentation for measuring excited state lifetime (see ref 24 for an overview of the development of time-resolved instrumentation), these values were estimated from the polarization. Obviously, the more viscous the solvent, the slower will be the rotation and again the higher will be the polarization. When the fluorescent ligand is bound to a slowly rotating macromolecule, such as a protein, then the observed polarization will increase over that for the probe free in solution. Hopefully, these considerations afford a clear qualitative picture of the relationship between polarization and fluorophore size and lifetime.

3.3.2. Quantitative Treatment: Perrin Equation

We may now consider fluorophore rotation in a more quantitative manner. Depolarization of the emission occurs if the fluorophore, and hence the emission dipole, rotates through an angle ω between the moment of excitation and emission. In fact

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(\frac{2}{3\cos^2\omega - 1}\right) \tag{8}$$

where P is the observed polarization. So, the total depolarization is determined by an intrinsic factor (P_0) and an extrinsic factor (ω). The multiplicative nature of the depolarization term, $2/(3\cos^2\omega - 1)$ was first pointed out by Soleillet.[25] Perrin related the observed polarization to the excited state lifetime and the rotational diffusion of a fluorophore.[13] Specifically, in his seminal paper, he derived the famous equation

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{RT}{\eta V}\tau\right) \tag{9}$$

where P is the observed polarization, P_0 is the limiting or intrinsic polarization, V is the effective molar volume of the rotating unit, R is the universal gas constant, T is the absolute temperature, η is the viscosity, and τ is the excited state lifetime.

We can rewrite this equation as

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right) \tag{10}$$

where ρ is the Debye rotational relaxation time, which is the time for a given orientation to rotate through an angle given by the arccos e^{-1}, which is 68.42°.

For a spherical molecule

$$\rho_0 = \frac{3\eta V}{RT} \tag{11}$$

For a spherical protein, it follows that

$$\rho_0 = \frac{3\eta M(\upsilon + h)}{RT} \tag{12}$$

where M is the molecular weight, ν is the partial specific volume, and h is the degree of hydration. A useful rule of thumb for estimating the rotational relaxation time for a

molecule (in solutions of low viscosity such as water) is to divide its molecular mass by 1000, which then gives, very approximately, the rotational relaxation time in nanoseconds. For example, fluorescein has a molecular mass around 330 Da, so we would expect its rotational relaxation time to be roughly 0.33 ns. A protein of molecular mass of 100,000 Da, on the other hand, would have a rotational relaxation time of roughly 100 ns. These estimates are crude and do not take into account the shape of the molecule, which can have profound effects on its rotation, but they certainly provide "ballpark" estimates, which can be very useful. For example, if we plug some rough numbers for fluorescein into eq 10, such as 0.33 ns for ρ, 4 ns for τ, and 0.5 for P_0, we can estimate a polarization of 0.016 for fluorescein in water, which is certainly in the ballpark of the measured value of 0.022.

From eq 10, one can see that a change in any of the three parameters, τ, ρ, or P_0, will result in a change in the polarization. Experimentally, τ can change depending on the probe environment, with changes often associated with a shift from a hydrophilic to hydrophobic environment, or vice versa. Changes in ρ are generally associated with a change of the size of the rotating particle, for example through changes in the aggregation state, association/dissociation with another molecule, or proteolysis. Changes in the P_0 at a fixed excitation wavelength are uncommon (discussed below), but changes in the effective P_0 can occur when energy transfer is present, resulting in a measured P_0 that is lower than the true P_0. For example, consider a protein with multiple probes attached such that there is energy transfer between them. The measured polarization will be lower than that of the same protein but without energy transfer because of the effect on the P_0. From eq 7, the P_0 is determined from the angle between the absorption and emission dipoles. In the case of energy transfer, the emission dipole is on another molecule; hence, there is generally a very large angle between the two dipoles.

3.4. Rotational Relaxation Time versus Rotational Correlation Time

We should note that it is not uncommon to see the term "rotational correlation time", often denoted as τ_c, used in place of the Debye rotational relaxation time. The information content of these terms is identical, since $\rho = 3\tau_c$, but we have observed that some people become rather fervently attached to the use of one term or the other. In the original development of the theories of rotational motion of fluorophores, Perrin and others used the rotational relaxation time, as originally defined by Debye in his studies on dielectric phenomena. Only later (in the 1950s) during the development of nuclear magnetic resonance was the term rotational correlation time used by Bloch. It thus seems reasonable for fluorescence practitioners to use ρ, but certainly adoption of either term should not lead to confusion. In terms of anisotropy and rotational correlation times, the Perrin equation would be

$$\frac{r_0}{r} = \left(1 + \frac{\tau}{\tau_c}\right) \tag{13}$$

3.5. Effect of Local Probe Mobility

In the case of fluorescence probes associated noncovalently with proteins (for example, porphryins, FAD, NADH, ANS, or fluorescent-antigen/antibody complexes), the probe is typically held to the protein matrix by several points of attachment, and hence, its "local" mobility, that is, its ability to rotate independent of the overall "global" motion of the protein, is restricted. In the case of a probe attached covalently to a protein, via a linkage through an amine or sulfhydryl groups, for example, or in the case of tryptophan or tyrosine side chains, considerable "local" motion of the fluorophore can occur. Such local probe mobility will result in a lower polarization than expected from consideration of the probe lifetime and the size of the protein. One of the first demonstrations of local probe motion, i.e., motion independent of the overall motion of the macromolecule to which the probe is associated, was by Gottleib and Wahl,[26] who characterized dansyl conjugates of synthetic polymers. Wahl and Weber[27] characterized in detail the dansyl conjugates of γ-globulins and demonstrated that isothermal variation of solvent viscosity, e.g., by addition of sucrose, gave rise to nonlinear plots of $1/P$ versus T/η (now usually termed Perrin−Weber plots). If a probe's rotational mobility is rigidly linked to the protein motion, the resulting Perrin−Weber plot would be expected to be a straight-line, as illustrated in Figure 9. An example of such a lack of local probe mobility was shown for the case of protoporphyrin IX associated with apohorseradish peroxidase.[28] If the probe is free to enjoy local probe mobility, however, then the slope of the Perrin−Weber plot will increase at higher viscosities (lower T/η values), as illustrated in Figure 9. In protein systems, one may, in fact, expect a hierarchy of probe mobilities, as illustrated in Figure 10. Probe local motion has sometimes been termed the "propeller effect", a misleading name which implies that probe mobility is isotropic in three dimensions. In fact, in many cases, local probe mobility may be anisotropic, since the barriers to movement are unlikely to be symmetrically arranged around the probe. Such anisotropic probe mobility was demonstrated in one system by Vandermeulen et al.[29] If one suspects that such local probe mobility is significantly decreasing the polarization/anisotropy in a system, Perrin−Weber plots are useful. Alternatively, one can carry out time-resolved anisotropy (or dynamic polarization) to quantify the rotational modalities of the system.[24] Verification of the extent of probe mobility can be important, since it is also possible that lower polarizations are due to lower than expected limiting

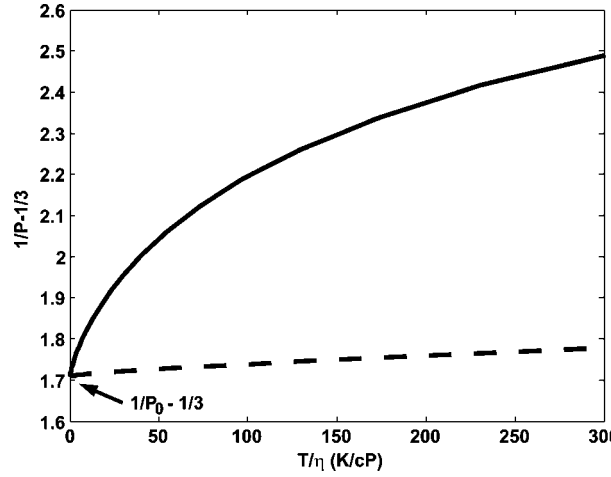

Figure 9. Perrin−Weber plot of a labeled spherical protein of 25 kDa with no local motion of a 4.0 ns probe (dashed line) and with 40% of the anisotropy due to rapid local probe motion (solid line). P_0 can be determined from the y-axis intercept.

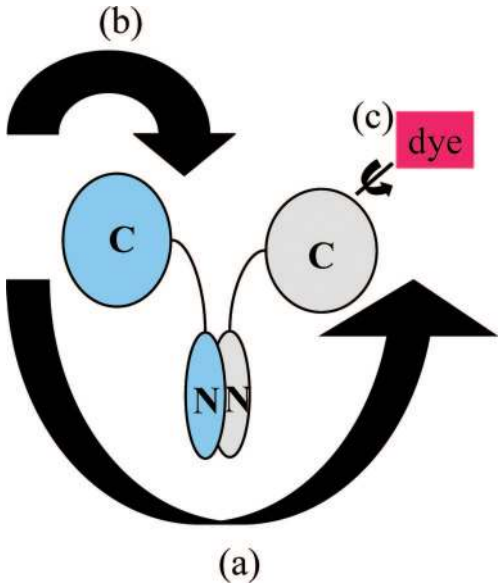

Figure 10. Illustration of different rotational modalities of a fluorescently labeled protein showing (a) global protein motion, (b) protein subunit flexibility, and (c) local probe motion.

polarizations. For example, as discussed below, the polarization spectrum of fluorescein can vary significantly depending on the nature of the conjugation chemistry linking it to another molecule.

4. Applications of Fluorescence Polarization/Anisotropy

4.1. General

As mentioned in the Introduction, fluorescence polarization methods are widely used in the clinical and biomedical fields. It is beyond the scope of this review to cover all applications. We shall focus on the most popular applications, which include ligand binding, immunoassays, and assays related to drug discovery.

It could be argued that drug discovery is the pharmaceutical industry's *raison d'etre*. Completion of the human genome project was expected, by many, to provide a significant impetus to this endeavor. However, discovery of promising compounds with positive therapeutic benefits is still a very challenging and time-consuming endeavor. The number of potential molecules to be screened is astronomical, and between natural sources and combinatorial chemistry methods, huge libraries of compounds exist, often comprising hundreds of thousands up to millions of compounds. Obviously, rapid high-throughput screening methods are required for the task. High-throughput screening using fluorescence polarization/anisotropy is becoming increasingly popular, since the appearance of commercial plate readers capable of handling not only the standard 96 well plate but also the 384 and 1536 formats (and no doubt soon 3072 and higher formats). Since libraries to be screened can be so large, the emphasis is on speed and accuracy. Excellent reviews of this area have been presented by Owicki[30] (covering up to the year 2000) and Smith and Eremin[31] (covering up to 2008), and those with a keen interest in this topic should refer to these articles.

4.2. Fluorescence Polarization/Anisotropy and Ligand Binding

Laurence, working with Weber's original instrumentation, first applied fluorescence polarization to study the binding of small molecules to proteins.[32] In particular, he studied the binding of various xanthene, acridine, and naphthalene dyes to bovine serum albumin (BSA). This work presaged much of the modern applications of fluorescence polarization to clinical chemistry. Specifically, it demonstrated that binding isotherms could be obtained without separation of the free and bound components. In modern parlance, we would say that Laurence had developed a homogeneous assay. A brief introduction to the use of polarization/anisotropy in binding assays was provided by Jameson and Sawyer.[33]

4.3. Additivity of Polarization/Anisotropy

The Perrin relationship was extended by Weber to consider ellipsoids of revolution with fluorophores attached in random orientations.[1] In this study, Weber also explicitly derived the relationship governing additivity of polarizations from different species, namely

$$\left(\frac{1}{\langle P\rangle} - \frac{1}{3}\right)^{-1} = \sum f_i\left(\frac{1}{P_i} - \frac{1}{3}\right)^{-1} \qquad (14)$$

where $\langle P\rangle$ is the observed polarization and P_i and f_i are the polarization and fractional contributions of the i^{th} species. (Note: The term "fractional contribution" actually refers to the fractional contribution to the photocurrent (or number of photons), which each particular molecular species provides. This contribution does not then strictly refer to the fraction of molecular species present but will depend upon the absorption and fluorescence spectra of each component, the particular region of the spectrum being monitored, and the response characteristics of the instrument at the relevant wavelength regions.) Weber's original motivation for deriving this expression was to understand how to treat polarizations associated with probes oriented along different rotational axes, in the case of proteins which behaved hydrodynamically as ellipsoids of revolution.

As mentioned above, in 1960 Jabłoński[22] defined the term anisotropy and pointed out that anisotropy was directly additive as:

$$\langle r\rangle = \sum f_i r_i \qquad (15)$$

This additivity, of course, follows directly from the law of additivity of polarization already derived by Weber in 1952. Perrin had actually described the important term $2P/(3 - P)$ in 1936[34] but had not given this function a name. Interestingly, Jabłoński did not reference Perrin's earlier work on this function, nor did he mention Weber's additivity of polarization equation, which appeared 8 years before the anisotropy equation (for a discussion of this point of attribution of significant photophysical work and for a marvelous account of the history of photophysics, the reader is directed to Bernhard Nickel's excellent series of papers[35–37]). An appreciation of the additivity of polarization and/or anisotropy is, of course, required if one wants to convert the observed polarization/anisotropy to molecular species, such as the fraction of a ligand bound (to determine a dissociation constant—*vide infra*), or to quantify substrate to product transformation in a process such as proteolysis.

4.4. Effect of Quantum Yield

The quantum yield of a fluorophore is defined as the ratio of the number of photons emitted to the number of photons absorbed.[18] The maximum quantum yield of a fluorophore is thus unity or 100%. Fluorophores with high quantum yields are obviously sought after for most applications in chemistry or biology, which is one reason why xanthene-based probes such as fluorescein and rhodamine are so popular. Fluorescein can reach a quantum yield of 0.92 while rhodamine 101 can reach a quantum yield of 1 (for a discussion of quantum yields and tables of some values, see Valeur,[18] Magde,[38] the classic work by Melhuish,[39] and a marvelous recent review by Rurack[40]). Although some probes do not significantly alter their quantum yield upon interaction with proteins, one should not take this fact for granted and it is advisible to check. If the quantum yield does in fact change, one can readily correct the fitting equation to take the yield change into account. In terms of anisotropy, the correct expression relating observed anisotropy (r) to fraction of bound ligand (x), bound anisotropy (r_b), free anisotropy (r_f), and the quantum yield enhancement factor (g—which can be found from the total intensity of the emission, i.e., $I_{||}$ and $2I_{\perp}$) is[41]

$$x = \frac{r - r_f}{r_b - r_f + (g - 1)(r_b - r)} \tag{16}$$

In terms of polarization, this equation is

$$x = \frac{(3 - P_b)(P - P_f)}{(3 - P)(P_b - P_f) + (g - 1)(3 - P_f)(P_b - P)} \tag{17}$$

A typical plot of polarization versus ligand/protein ratio is shown in Figure 11 (data from ref 42). In this experiment, 1 μM mant-GTPγS (a fluorescent, nonhydrolyzable GTP analogue) was present and the concentration of the GTP-binding protein, dynamin, was varied by starting at high concentrations followed by dilution. The binding curve was fit to the anisotropy equation (in this case, the yield of the fluorophore increased about 2-fold upon binding). A K_d of 8.3 μM was found. If one did not take into account the enhancement of the mant-GTPγS fluorescence upon binding, one would erroneously determine a K_d near 2.5 μM.

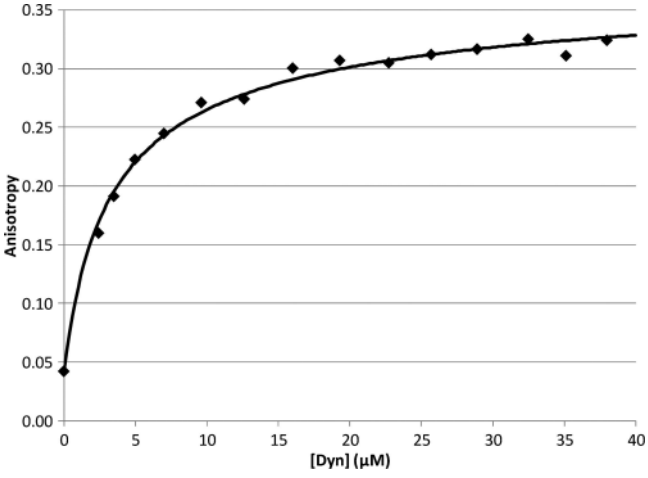

Figure 11. Binding isotherm of mant-GTPγS (a slowly hydrolyzable GTP analogue) to the large GTPase dynamin. The enhancement of the fluorescence of the mant-GTPγS upon binding was approximately 2-fold, and the K_d was determined to be 8.3 μM. If one were to ignore the enhancement of the mant-GTPγS, the K_d would erroneously be determined as 2.5 μM. Reprinted with permission from ref 44. Copyright 2005 Elsevier.

In the 1960s and 1970s, fluorescence polarization began to be more frequently applied to protein−ligand interactions. Examples of the early studies include that by Chien and Weber,[43] who studied the binding of a fluorescent ATP analogue (ethenoATP) to aspartate transcarbamylase. One of the early direct applications of fluorescence polarization to clinical chemistry was the work of Spencer et al.,[44] who described an automated flow-cell instrument.

4.5. Background Subtraction

We should note that correction of polarization/anisotropy measurements for background contributions cannot be done simply by subtraction of the background polarization/anisotropy from that of the sample. Rather, the intensities corresponding to the parallel and perpendicular components of the background must be subtracted from the respective intensities of the sample polarization/anisotropy. If this procedure is carried out carefully, polarization values can be extracted even from significant background levels. For example, Jameson et al.[45] were able to resolve polarization values for 100 femtomolar fluorescein in the presence of a substantial contribution from Raman scattering. This type of extreme background correction using photon counting methods requires sufficient sample counts so that the background corrected signal will still have enough counts to allow reasonable precision (*vide infra*).

4.6. Sources of Errors

The most fundamental source of error in fluorescence polarization/anisotropy determinations would be actual errors in the polarization measurements themselves. In the days when equipment was home-built, i.e., before the commercial availability of fluorescence polarization instruments, one had to be particularly aware of such considerations. For better or worse, researchers now usually trust the manufacturer to provide an accurate instrument. However, a sound knowledge of the potential sources of errors in the instrument is always useful. Before considering the more subtle issues, we should remind novices that a polarization bias may be introduced to measurements of $I_{||}$ and $I_{\perp}$ by monochromators. This polarization bias is manifested by a change in either $I_{||}$ or $I_{\perp}$ depending on the geometry of the instrument and is corrected using the so-called "G factor", usually attributed to Azumi and McGlynn.[46] G is given by the equation

$$G = I_{HV}/I_{HH} \tag{18}$$

where I_{HV} is the intensity with the excitation and emission polarizers oriented horizontally and vertically (with respect to the laboratory axes), respectively. For I_{HH}, the excitation and emission polarizers are both oriented horizontally. The correction required for emission collected through a monochromator can be significant and varies substantially as a function of wavelength. Thus, the polarization can be determined from the formula

$$P = \frac{I_{||} - GI_{\perp}}{I_{||} + GI_{\perp}} \tag{19}$$

Gregorio Weber presented a careful and detailed consideration of potential sources of errors in polarization measure-

ments.[47] As pointed out by Weber, in order to measure a polarization of 0.05 with a precision of 1%, $I_{\parallel}$ and $I_{\perp}$ must be known to within 1 part in 1000. Weber stated that systematic errors in polarization measurements may be generally placed into three categories, namely:

(1) Errors resulting from faulty settings of the parts (by which he specifically meant the orientation of the polarizers).

(2) Errors resulting from the non-negligible size of the source (by which he meant the aperture effect (see ref 21 for a recent discussion of this effect)).

(3) Errors resulting from stray light (see ref 21 for a recent discussion).

Considerations of the inherent precision of polarization measurements and photon counting statistics were discussed by Jameson et al.,[45] in their description of the first photon-counting polarization instrument. More recently, Jameson and Mocz[41] extended these discussions of measurement errors by explicitly considering the effect of specific errors or uncertainties on the final estimate of the fraction of bound ligand and, hence, on the estimated dissociation constant of the ligand/protein complex. An important finding was that uncertainties in the estimated or experimental value of the anisotropy of the bound probe cause the largest inaccuracies in the output dissociation constants. This consideration may be especially important for large scale screening experiments, where a number of unknown proteins are tested simultaneously. Assuming the same anisotropy for the bound probe in all systems may lead to large inaccuracies in the determination of binding constants. Other input parameters such as the anisotropy of the free ligand, the fluorescence enhancement factor, and the precision of anisotropy readings propagate smaller but not insignificant uncertainties into the results. The uncertainty in the enhancement factor has a complex effect when it is jointly present in combination with uncertainties from other factors. We note that one of the primary motivations for this error analysis study was the fact that, in the recent literature—in particular the literature associated with clinical chemistry applications of the polarization/anisotropy method—some investigators analyze their polarization data using the formulation associated rigorously only with the anisotropy function (see, for example, ref 48). This practice leads to systematic deviations in determination of the fraction bound and subsequently of the dissociation constant, K_d. Specifically, direct substitution of polarization values into eq 15, derived for anisotropy measurements, instead of converting polarization values to anisotropies using eq 5 or 6, or instead of using eqs 14 or 17, derived for polarization measurements, results in *systematic overestimation of x and underestimation of* K_d. This approach is, of course, intrinsically wrong but has been justified by the rationalization that the attendant errors are not large.

5. Fluorescence Polarization Immunoassays (FPIA)

The application of fluorescence polarization to study antigen−antibody interactions was first developed by Walter Dandliker in the early 1960s. In the initial experiments by Dandliker and Feigen,[49] ovalbumin was labeled with fluorescein using fluorescein isothiocyanate and then antibodies were raised to the fluorescein−ovalbumin adduct. Polarization and intensity measurements were then used to study the binding of this antibody−antigen system. This early work was followed by a series of similar studies which laid the groundwork for much of the clinical chemistry and high-throughput studies which followed over the next decades. As pointed out by Dandliker, and by generations of practitioners after him, the fluorescence polarization immunoassay (FPIA) is homogeneous; that is, it requires no separation of free and bound material. Rather, it relied upon Weber's principle of additivity of polarization (*vide supra*). The basic principles underlying a polarization immunoassay are illustrated in Figure 12A. These may be summarized as the following:

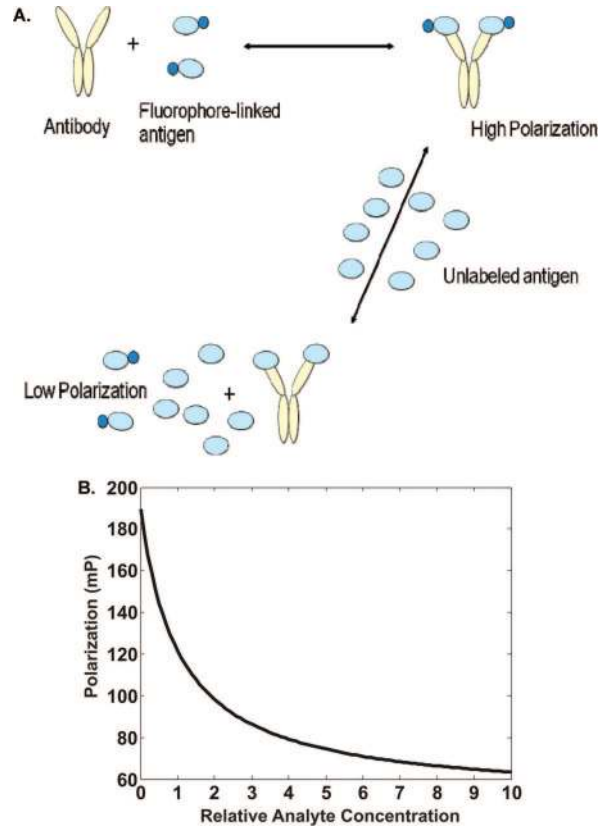

Figure 12. (A) Scheme of a fluorescent immunoassay. An antibody and fluorescently labeled antigen are mixed and allowed to equilibrate, and then the unlabeled analyte or antigen is added, which displaces the labeled antigen, resulting in a lower polarization. (B) Plot depicting a fluorescence polarization immunoassay; the free ligand has a polarization of 50 mP, while the bound ligand has a polarization of 190 mP.

(a) Add a fluorescent analogue of a target molecule—e.g., a drug—to a solution containing antibody to the target molecule.

(b) Measure the fluorescence polarization, which corresponds to the fluorophore bound to the antibody.

(c) Add the appropriate biological fluid, e.g., blood, urine, etc., and measure the decrease in polarization as the target molecules in the sample fluid bind to the antibodies, displacing the fluorescent analogues.

The type of plots resulting from this approach is illustrated in Figure 12B.

5.1. Early Work—1960s and 1970s

As mentioned above, the first polarization immunoassay was carried out by Dandliker and Feigen in 1961.[49] This study was an initial proof of principle utilizing ovalbumin conjugated with FITC reacting with antibodies raised against this adduct. Soon after, in 1962, Haber and Bennett[50] used fluorescence polarization to follow the binding of FITC-

labeled insulin (B-chain), RNA, and BSA with their respective antibodies (note in this study antibodies were raised against protein that were not conjugated with FITC—unlike the approach of Dandliker and Feigen). Interestingly, Haber and Bennett commented that Dandliker and Feigen excited their samples using the 436 nm line of a mercury lamp whereas they themselves used 495 nm excitation, a difference which they speculated could affect the polarization values. As shown in the excitation polarization spectrum of FITC, the limiting polarization upon 436 nm excitation is indeed significantly lower than that at 495 nm excitation. In 1965, Dandliker et al.[51] studied antipenicillin antibodies using FITC polarization. An interesting early paper by Spencer et al.[44] described an automated flow-cell polarization instrument designed primarily to measure enzyme−inhibitor and antibody−antigen interactions using fluorescence polarization. This instrument was designed by Spencer, who had been a graduate student with Weber and who, at the time of the instrument construction, was still a postdoctoral fellow in Weber's lab. This instrument was the first of its type targeted to clinical chemistry and polarization and essentially marked the start of SLM Instruments, Inc., which became the premier fluorescence instrument manufacturer of its time.

In 1976, Watson et al.[52] developed a fluorescence polarization immunoassay for gentimycin, a broad-spectrum antibiotic. In 1978, Urios et al.[53] described a fluorescence polarization immunoassay for human chorionic gonadotropin (HCG). In this study, the HCG was carbamylated and reacted with FITC. Although the change in polarization was not large, ~0.30 to ~0.35 for FITC-HCG in the absence and presence of antibody, respectively, the precision of the measurement (±0.003) was high. Although the immunological reactivity of the HCG was reduced ~30% by the FITC linkage, the polarization results compared very well with the standard hemeagglutination inhibition assay. Moreover, the rapidity of the polarization method recommended its use in certain clinically urgent circumstances. In 1978, McGregor et al.[54] designed a fluorescence polarization immunoassay for phenytoin, a common antiepileptic drug, in serum to replace the time-consuming standard method, gas−liquid chromatography. In 1979, Kobayashi et al.[55] described a polarization immunoassay for serum cortisol using FITC linked to cortisol 21-amine. Interestingly, in this paper the polarization axis scale presented ranged from 100 to 400 and was labeled "arbitrary units." Of course, polarization units are not "arbitrary" although they are dimensionless. In fact, this use of standard polarization units times 1000 presaged the present day standard of "millipolarization" units introduced formally in 1981 by Jolley et al.,[56] in the paper describing the Abbott TDx system (*vide infra*). Millipolarizations, or mPs, pronounced "millipees", are almost ubiquitous in the clinical chemistry literature.

5.2. Abbott Laboratories and the TDx

In the early 1980s, Abbott Laboratories introduced the TDx instrument, originally to monitor the levels of therapeutic drugs in serum. In fact, one of us (D.M.J.) recalls when representatives from Abbott laboratories visited Weber's laboratory in the late 1970s to discuss fluorescence polarization measurements. In a series of publications, the basic principle of the TDx approach, using fluorescence polarization, was demonstrated using several different target molecules modified by addition of fluorescein, including aminoglycoside antibiotics, theophylline, and phenolbarbitol.[56−59] During the 1980s, many more assays were developed for the TDx platform, including assays for drugs of abuse, for toxicology, and for endocrinology related issues, and others.

5.3. Recent Work

In the past few decades, a great many FPIAs were developed in many laboratories for a myriad of research problems, and we shall not attempt to provide a comprehensive list. Excellent reviews of many of these assays have been provided. For examples, see Smith and Eremin,[31] Eremin and Smith,[60] Nasir and Jolley,[61−63] Jolley et al.,[64,65] Sportsman,[66] Sportsman et al.,[67] and Owicki.[30]

6. Other Polarization Assays

Fluorescence polarization/anisotropy methods have been applied to a large number of biochemical systems. Many assays have been developed to assess the activities of diverse enzymes, such as proteases, kinases, phosphatases, etc. We shall consider some of these systems in this section.

6.1. Protease Assays

Proteolytic processing, mediated by proteolytic enzymes or proteases, is critical to many vital biological processes, including post-translational protein processing, blood clotting, digestion, hormone processing, apoptosis, and many others, as well as many deleterious processes, such as those mediated by anthrax and botulinum neurotoxins. Hence, an evaluation of protease activity is often a requirement for an understanding of a particular pathway or for development of novel therapeutic agents. Protease assays have been around for many decades, but more recently, the development of rapid and sensitive protease assays suitable for high-throughput screening has attracted considerable attention. Fluorescence polarization lends itself very well to such assays, since the essential aspect of a protease is to cleave a peptide bond, which almost always results in smaller molecular weight species. Hence, if the target protein can be labeled with a fluorescent probe, one would expect the polarization to decrease after proteolysis, since the fluorophore will be able to rotate more rapidly after the protein mass to which it is tethered is reduced in size (Figure 13A).

6.1.1. Traditional Approaches

One of the earliest examples of a polarization protease assay was presented in 1973 by Spencer et al.,[44] using the automated fluorescence polarization instrument mentioned in section 5.1. In their assay, the target proteins, casein or insulin, were labeled with FITC and the polarization was followed after introduction of trypsin. The assays were repeated in the presence of known amounts of trypsin inhibitor. Once suitable standard curves were developed for the trypsin inhibitor, serum samples were studied to evaluate the levels of trypsin inhibitor as well as α_1-antitrypsin in patients. In 1978, Maeda[68] published studies of the use of fluorescence polarization to study proteolysis of FITC-labeled proteins by papain, trypsin, Pronase, and pepsin. Since these early works, a great many fluorescence polarization protease assays have appeared—we shall describe a few representative examples in chronological order to give a feeling for the direction of the field.

(1) Bolger and Checovich[69] described a polarization based assay for serine proteases, sulfhydryl proteases, and acid

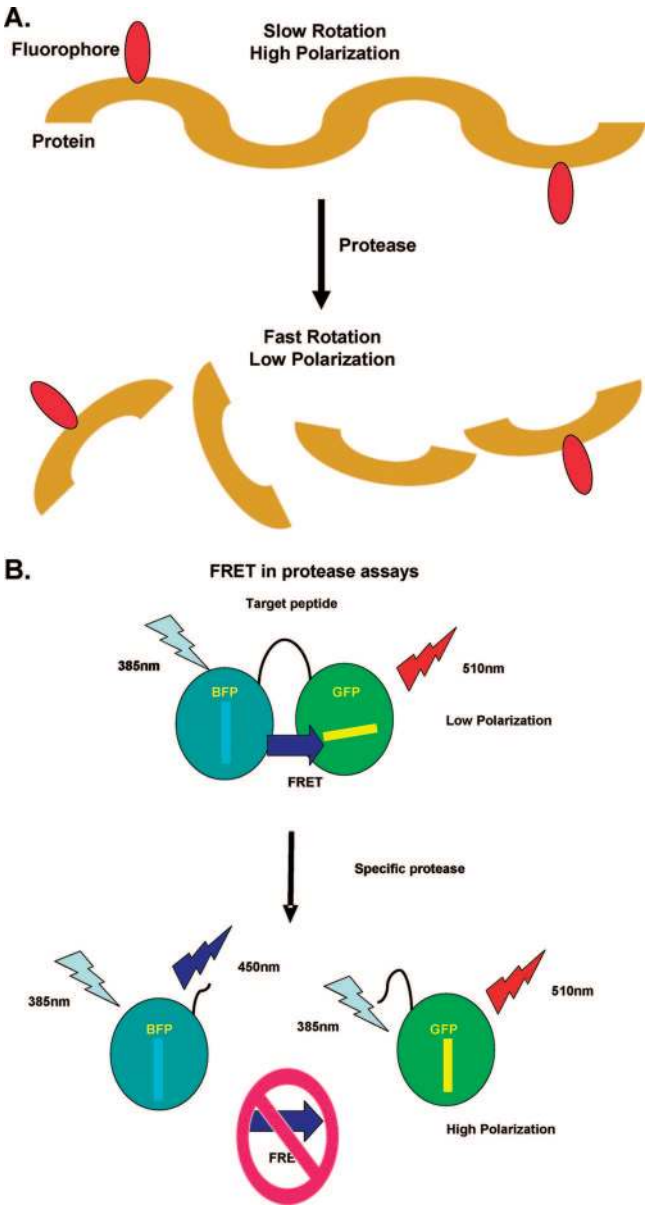

Figure 13. (A) Illustration of the typical protease assay, where the polarization decreases following proteolysis. (B) Illustration of a DARET assay in which the polarization is initially low due to FRET from the photoselected Blue Fluorescent Protein (BFP) to the Green Fluorescent Protein (GFP) acceptor; the polarization increases following proteolysis due to the direct photoselection and excitation of the GFP.

proteases wherein a fluorescein thiocarbamoyl-casein substrate is cleaved to form smaller fluorescein labeled peptides. To avoid the issue of altered fluorescence properties of fluorescein at low pH, they removed an aliquot of the sample at specific time points and diluted it into a strongly buffered solution at pH 8.8.

(2) Schade et al.[70,71] used BODIPY-α-casein as a substrate for polarization protease assays, as its fluorescence properties are less pH sensitive than those of fluorescein and it shows a larger change in the polarization around neutral pH. They used the BODIPY-α-casein substrate with purified proteases, and dental and bacterial cultures which excrete proteases.

(3) Grys et al.[72,73] got their teeth into the same assay system as Schade et al.,[74] and they reported a decrease in protease activity with scaling and root-planing. They analyzed 208 subgingival samples with 87 containing detectable protease activity, with protease activity decreasing when measured 8 months after scaling and root-planing.

(4) Levine et al.[75] used a short polypeptide with a biotin at the C terminus and fluorescein at the N terminus to assay human cytomegalovirus protease in a 96 well plate. After a predetermined time, tetrameric avidin was added to the reaction mix, serving two purposes: first, the avidin bound to the biotin increased the molecular mass and, hence, polarization of the uncleaved substrate, and second, the avidin bound substrate was not cleaved by the protease.

(5) Liu et al.[76] modified a single chain variable fragment from an antibody to the small histidine containing protein HPr into a site specific protease and followed the kinetics of the proteolysis using fluorescein labeled HPr. They also investigated the binding affinity of the original single chain variable fragment and its mutants, and the effect of pH on the proteolysis.

(6) Simeonov et al.[77] investigated the cleavage of a short polypeptide by cathepsin G using an assay involving the addition of polyarginine, which binds the substrate and reaction products with different affinities, and adjusted the ionic strength of the buffer such that their method can be applied when both the substrate and product are negatively charged.

(7) Blommel and Fox[78] took advantage of recombinant DNA technologies to develop a range of fluorescent substrates using the FLAsH tag (tetraCys motif) approach,[79] which allowed them to introduce the fluorophore to a specific location in the target protein. In this work the FLAsH tag motif was inserted into short peptide sequences attached to maltose binding protein. Each sequence was the specific substrate for a particular protease, namely trypsin, enterokinase, factor Xa, thrombin, and tobacco etch virus (TEV). Polarization/anisotropy experiments were carried out using a 384-well format, which allowed them to follow the kinetics of the reactions.

(8) Lee et al.[80] investigated the proteolytic properties of the parasitic protozoan ciliate *Uronema marinum* on a fluorescein labeled casein substrate. They incubated the fluorescein-casein substrate with live cells of *U. marinum* in 96 well plates and followed the polarization at numerous time points. From this work they determined that the main proteases excreted by the organism were metalloproteases.

(9) Kim et al.[81] combined microfluidics with polarization to monitor the cleavage of tetramethylrhodamine-α-casein by a variety of proteases with the eventual goal of expediting the sequential measurement of time dependent enzymatic assays and inhibitor screening.

(10) Cleemann and Karuso[82] used epicocconone (a naturally fluorescent compound from the fungus *Epicoccum nigrum*[83]) as the fluorophore for their protease polarization assay. Epicocconone binds reversibly to proteins to form a highly fluorescent internal charge-transfer complex. Due to the complex photophysics surrounding the fluorescence of epicocconone, it is favorable to use fluorescence polarization to follow the protease kinetics rather than the fluorescence intensity.

(11) Chen et al.[84] investigated the binding of BODIPY FL-pepstatin A to cathepsin D and the effect of varying pH and addition of other proteases.

6.1.2. FRET-Based Approach to Polarization

As mentioned earlier in section 2.2.3, one of the earliest demonstrations of FRET was the observation in 1924 by

Gaviola and Pringsheim[14] that the polarization of fluorescein in viscous solvents decreased as the concentration of the dye increased. Theories of depolarization after such self-transfer (also termed homotransfer) were given by Weber[85] and several other researchers (reviewed in Vandermeer et al.[86]). Depolarization due to heterotransfer, i.e., between different molecular species, was also observed years ago. Among the earliest reports of this phenomenon was Weber's observation of depolarization of tryptophan fluorescence in proteins due to FRET from excited tyrosine residues.[87] Although most heteroFRET experiments rely on changes in intensity or lifetime, it is also possible to utilize changes in polarization to monitor changes in FRET efficiency. Most FRET measurements with Fluorescent Protein systems (e.g., GFP etc.) have been done using fluorescence intensity, often using the ratio of intensities taken at two wavelengths, e.g., the emission maxima of the donor and acceptor. The first FRET based protease experiment using Fluorescent Proteins was reported in 1996 by Mitra et al.,[88] who made a construct wherein BFP and GFP were connected by a 20-amino acid linker containing the substrate sequence for Factor Xa. Another example of such an assay is one described in U.S. Patent 7399607[89] and World patent WO/2007/047342[90] (and presented at symposia[91−93]), which details a proteolytic assay of a GFP-SNAP25-BFP substrate, wherein the SNAP25 derived peptide contains the cleavage site for Botulinum Neurotoxin type A (BoNT/A). The concept underlying this assay is illustrated in Figure 13B. The substrate has a polarization value near zero when intact and near 0.40 following cleavage. The low polarization of the substrate is due to FRET between the BFP and the GFP. In this approach, the BFP is preferentially excited by illumination at ~380 nm and emission from the GFP moiety is observed. The large angle between the BFP absorption dipole and the GFP emission dipole results in a very low initial polarization. After cleavage, only directly excited GFP is observed, which—even at excitation near 380 nm—has a large polarization (Figure 19). Hence, the large increase of the polarization upon cleavage of this substrate is the result of two main factors: first the high efficiency of FRET from the BFP to the GFP and second the large angle between the two chromophores. By following the kinetics of the assay, one can determine the rate of cleavage by the BoNT/A under different conditions and calculate the turnover rate of the substrate. Note that since this assay involves FRET, there is also a change in the intensity as the assay progresses and calculation of the fraction of intact and cleaved substrate must take this into account using eq 16 or 17. This combination of fluorescence polarization and FRET, termed DARET for Depolarization After Resonance Energy Transfer, promises to be a useful addition to the armamentarium of protease aficionados.

6.2. Kinase Assays

The importance of kinases in a myriad of cell signaling pathways, e.g., the mitogen-activated protein (MAP) kinases which are involved in the regulation of many oncogenes, makes kinase inhibitors attractive targets in drug discovery screening. Fluorescence polarization assays have been developed for many diverse kinases, and we shall discuss a few to illustrate some of the concerns and the approaches being utilized. A number of fluorescence polarization kinase assays are, of course, commercially available.

(1) Fowler et al.[94] evaluated the use of fluorescence polarization in high throughput screening for inhibitors of two kinases, namely, c-jun N-terminal kinase (JNK-1) and protein kinase C. Comparisons were made with standard [γ-^{33}P] ATP filter wash assays, and the interference from fluorescent compounds was evaluated. They also compared the steady-state fluorescence polarization results to a lifetime discriminated polarization approach.

(2) Protein tyrosine kinases, especially the Src family, are popular inhibitor targets in several cancer therapies. Newman and Josiah[95] compared several Src kinase assays, including those based on fluorescence polarization, time-resolved fluorescence resonance energy transfer (TR-FRET), and the dissociation enhanced lanthanide immunoassay method. The authors conclude that the fluorescence polarization and TR-FRET assays performed equally well but that the lanthanide-based system suffered from technical difficulties in this assay.

(3) Phosphoinositol signaling pathways are critical for numerous cell signaling pathways, and several disease states can result from disruptions of these pathways. Hence, the efficacy of drugs on phosphoinositol-modifying enzymes is of great interest. Drees et al.[96] describe competitive fluorescence polarization assays for the detection of phosphoinositide kinase and phosphatase activity using fluorescein, BODIPY, and tetramethylrhodamine-based derivatives of phosphoinositides. Specifically, the activity of phosphoinositol 3-kinase and the type-II SH2 domain containing inositol-5 phosphatase were studied.

(4) Turak-Ettien et al.[97] described the use of red-shifted dyes in a fluorescence polarization assay for the AKT kinase (also called protein kinase B), an inhibitor of apoptosis. By using very red-emitting dyes, specifically Cy3B and Cy5, the effects of autofluorescence and light scattering in cell extracts were reduced.

6.3. *Z*-Factor

When carrying out a high throughput screening assay, it is useful to have a measure of the confidence with which a compound makes a hit, i.e., interacts with the target system. Problems can arise if one only compares a single sample and control, as this method does not take into account the statistical variation associated with multiple repetitions, the background level, and experimental and instrument variations. A frequently used parameter to judge the effectiveness of an assay is the *Z*-factor, introduced in 1999 by Zhang et al.:[98]

$$Z = 1 - \frac{3\sigma_S + 3\sigma_C}{|\mu_S - \mu_C|} \tag{20}$$

where σ and μ are the standard deviation and mean, and the subscripts S and C denote the sample and control, respectively. According to Zhang et al.,[98] a Z value of 1 indicates an ideal assay with no variation among various runs, while $1 > Z \leq 0.5$ signifies an excellent assay with large separation of the sample and control. Z values around zero indicate essentially no difference between the sample and control, and screening with this type of assay is almost impossible. While the Z factor indicates the quality of a particular assay for high throughput screening (HTS), the Z' factor evaluates the overall quality of the assay in the absence of the test compounds.

$$Z' = 1 - \frac{3\sigma_{C+} + 3\sigma_{C-}}{|\mu_{C+} - \mu_{C-}|} \quad (21)$$

where σ_{C+}, σ_{C-}, μ_{C+}, and μ_{C-} are the standard deviation and mean of the positive and negative control samples, respectively. The Z and Z' factors are not used exclusively with polarization assays but have also been used extensively with assays based on other parameters such as fluorescence intensity,[99] absorptivity,[99] ratiometric fluorescence assays (i.e., with FRET),[100] and mass spectrometry.[101]

Typical Z' values for high throughput assays range from 0.7 to 0.9; see, for example, Vaasa et al.[102] (0.74), Peterson et al.[103] (0.76), Simeonov et al.[104] (0.84 and 0.91), Mathias et al.[105] (0.83−0.93), Cervantes et al.[106] (∼0.7), and Huang et al.[107] (0.76−0.89). Kozany et al.[108] developed an assay for the interaction of fluorescein labeled rapamycin and FK506 analogues with FK506 binding proteins with Z' values of >0.7 using polarization. The DARET assay discussed in section 6.1.2 gives a Z'-factor of ∼0.95. An interesting comparison of Z'-factors obtained for a 17β estradiol fluorescence polarization binding assay using either one or two-photon excitation was presented by Tirri et al.[109] An indication of the popularity of the Z-factor is the fact that the 1999 Zhang et al.[98] reference has been cited more than 1000 times to date!

7. Polarization and Imaging

7.1. Polarization Imaging Instrumentation

Polarization imaging can be used to combine the high spatial resolution provided by a microscope with the ability to interrogate the rotational mobility of the sample. The basic premise of the polarization microscope is similar to that of the polarizing fluorimeter, namely that the sample is excited with a particular polarization of light and the emission is resolved into parallel and perpendicular components. This approach has been achieved in different ways, but these can be classified into two general approaches: either each polarization component is spatially separated and measured simultaneously or the excitation or emission polarizer is rotated.

Separation of polarized components of the emission is most commonly achieved by the use of a polarizing beam splitter in the emission path, which sends each polarization component to a separate detector, usually a photomultiplier tube (PMT) or an avalanche photodiode (APD).[110] Alternatively, a Wollaston prism can be placed in the emission path to introduce an angular divergence of each polarization component. This approach can be used with either a camera, where each polarization component is imaged onto one-half of the camera[111] or onto a PMT, or APD if the beam is descanned. Sequential acquisition of each polarization component is achieved by rotating either the excitation or emission polarizer through 90°.[112] Alternatively, orthogonal polarizers can be mounted on a slider. Through the determination of each pair of polarization intensity ratios, one can determine the orientation of the fluorophore; for example, Axelrod used this approach to determine that 3,3′-dioctadecylindocarbocyanine (DiL) is oriented parallel to membrane surfaces.[113]

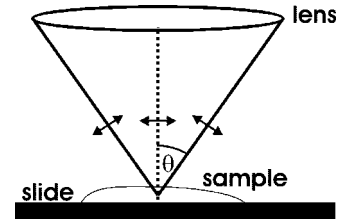

Figure 14. Diagram of light rays emitted from a lens focused on a sample with half angle θ. Arrows indicate the orientation of the electric field vector of the incident light.

7.1.1. Effect of Numerical Aperture

One important factor that should be considered in polarization measurements is the effect of the numerical aperture (NA) of the lenses in the instrument. Although these effects are most pronounced in microscopy applications, they can also give rise to inaccurate polarization values in normal spectrofluorimeters and plate readers. In normal research-quality spectrofluorimeters, the deviations of measured polarization values from the true values are only a few percent due to the collection optics, which subtend angles on the order of 15°; but it is interesting to note that Weber's original instrument had less error, since he used optics that subtended only ∼2.5°.[47] The trade-off, of course, is larger light collection efficiency versus more accurate polarization values.[21] The numerical aperture defines the angle over which a lens can accept or emit light, given by

$$\mathrm{NA} = n \sin(\theta) \quad (22)$$

where n is the refractive index of the surrounding media and θ is the half angle of the maximum cone of light that can be accepted or emitted from the lens. The polarization theory described above assumes that the excitation light is a plane wave incident on the sample, and similarly for the emission light. However, if the emission is collected through a lens and/or the excitation light is convergent on the sample, then a decrease of the measured polarization can occur. For example, in a fluorescence microscope, as the numerical aperture increases, the measured polarization decreases, with the measured polarization for tetramethylrhodamine in 95% glycerol decreasing from ∼0.45 to ∼0.3 for the case of NA = 0 (plane wave) to NA = 1.3.[114]

The scheme of the effect of the NA is shown in Figure 14. The excitation light from the lens fills a cone with angle θ. Since the electric field vector is perpendicular to the direction of propagation of the light, at the edges of the cone there is a component of the electric field which is perpendicular to the sample plane. This effect essentially influences the photoselection process of the fluorophores in the region of focus. Prior to the lens, all the electric field vectors were parallel, but once the light converges after the lens, the electric field vectors are no longer parallel, meaning that the light is no longer perfectly polarized. As the light does subtend a cone on the sample, this effect is more significant for the light rays toward the edge of the cone than for those in the center.

For polarization imaging conducted on a scanning microscope, such as a conventional laser scanning confocal microscope, the correction required due to the NA of the lens will vary within the image because the angle of the light rays with the sample plane will vary with each pixel. There are also many other factors which should be considered, including the effect of noncollimated light passing through

the polarizers and the polarization dependence on other optical elements in the microscope (e.g., plane and dichroic mirrors), among others. For detailed considerations of instrument artifacts affecting polarization imaging, see refs 113 and 115−117.

7.2. Applications

Axelrod[113] reported one of the first applications of fluorescence polarization applied to an imaging system. He investigated the orientation of carbocyanine in erythrocyte ghosts membranes and provided a rigorous mathematical treatment of the corrections needed for the high numerical aperture afforded by the objective lens onto the sample. From this work he determined the absolute orientation of the fluorophore with respect to the membrane surface. He cites work done previously by Dragsten, who also provides a treatment for the correction due to the numerical aperture, but this work appears to have been given during a seminar at a Biophysical Society meeting in 1977 and is contained only within Dragsten's Ph.D. thesis. Dragsten used the correction for the numerical aperture to investigate the dependence of the polarization of merocyanine 540 under different membrane potential conditions. However, even though these measurements were conducted through an objective lens, an image of the sample was not recorded. The theory of the correction for large numerical aperture required for high resolution imaging has recently been revisited and somewhat simplified by Fisz[117] through the application of their "symmetry adapted calibration" method.

Axelrod also extended the use of polarization imaging to include TIRF (total internal reflection fluorescence) microscopy with a detailed description of the effect of the different polarizations on the imaging.[118] Bos et al.[119,120] also developed a theory of polarized TIRF microscopy and applied it to determine the distribution of orientations of porphyrin and porphyrin-like molecules adsorbed to a surface.

Polarization imaging allows one to study cellular interiors and hence obtain detailed spatial information, in very small volumes, not available with *in vitro* studies. For example, Gough and Taylor[121] present a detailed polarization imaging study of the binding of fluorescently labeled calmodulin and myosin in fibroblasts. Their results showed regions of the cells which appeared to have the calmodulin bound, in particular in the leading lamellae and in the tails of highly polarized and migrating fibroblasts. In serum starved fibroblasts, when stimulated, the polarization of the calmodulin increased rapidly and then decayed rapidly (Figure 15A−D), consistent with the time course of the calcium response. Kho et al.[110] described a method for the linear unmixing of multiple compounds, which cannot be easily spectrally resolved, based on their polarization, and applied it to the case of fluorescent microspheres with and without energy transfer. Burghardt et al.[122] extended the use of polarization imaging to include TIRF microscopy, where they used it to investigate the population of angles subtended by a GFP-cardiac myosin regulatory light chain. They used the intrinsic property of polarization whereby only fluorophores with their absorption dipoles parallel to the excitation light electric field vector are excited and determined that the GFPs were oriented preferentially with the dipole nearly parallel to the fiber axis. Additionally there was a smaller population with their dipoles at a large angle to the fiber axis.

One application particularly suited to polarization imaging is the investigation of ordered structures, e.g. crystalline solids. Baumann and Lutz used standard fluorescence confocal microscopy of the photosensitive microvilli within the compound eye of *Drosophila* to investigate the twisting of rhabdomeres within the photoreceptor.[123] Baumann, in collaboration with colleagues at Carl Zeiss MicroImaging GmbH, used anisotropy imaging to visualize the orientation of actin filaments labeled with AlexaFluor488-phalloidin within the microvilli of the rhabdomeres (Figure 15E and F). Note: As mentioned earlier, in an ordered system, where all the fluorophore dipoles are aligned, the polarization may be greater than the limits achieved in solution, i.e. 0.5, because of the absence of averaging over the angular distribution of the fluorophores.

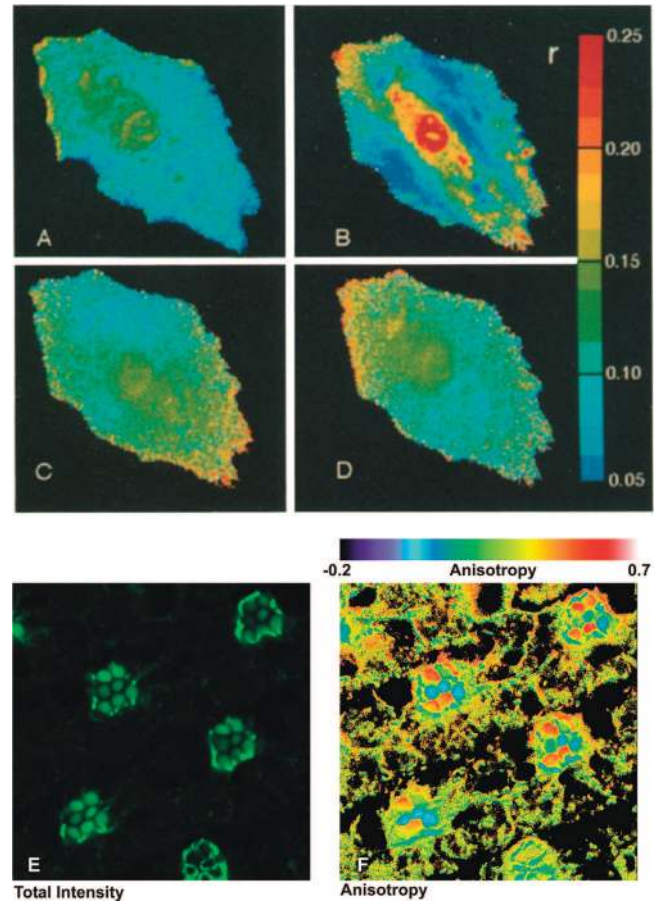

Figure 15. Steady state fluorescence polarization images of FITC-Calmodulin in serum starved fibroblasts—from ref 121: (A) Before stimulation and (B) 60 s, (C) 90 s, and (D) 120 s following stimulation with media containing serum. The color bar represents the measured anisotropy from 0.25 (red) to 0.05 (blue). Reprinted with permission from ref 121. Copyright 1993 the Rockefeller University Press. (E) Total intensity image and (F) anisotropy image of actin filaments labeled with AlexaFluor488-phalloidin in the drosophila eye. The anisotropy images, acquired on a LSM 710 with the anisotropy option, are courtesy of Otto Baumann from the University of Potsdam and Robert Hauschild, Eva Simbürger, and Oliver Holub from Carl Zeiss MicroImaging GmbH.

Davey et al.[124] used a suite of single photon and two photon microscopy techniques, including polarization imaging and fluorescence lifetime imaging microscopy (FLIM), to investigate the association of IgE and its receptor with cholesterol rich lipid rafts. Interestingly, they report that the lifetime of Alexa 488 bound to the IgE was shorter than that of the free probe in aqueous solution. Some groups have also measured the polarization of a microscopic sample without scanning using their microscope as a polarizing fluorometer. Borejdo and his colleagues used their micro-

scope with excitation and emission polarizers to measure the effect of ATP on the orientations of actin within muscle fibers[125] and of microvilli[126] and later used caged ATP molecules released via UV illumination to measure the polarization of actin bound myosin crossbridges.[127,128] By using caged ATP, only ~400 or fewer crossbridges were investigated within the focal volume. From this result they showed that the change of anisotropy was larger for a smaller number of observed crossbridges. In this series of studies, they excited with parallel and perpendicular light sequentially and measured the parallel and perpendicular intensities, which were subsequently averaged. Picart et al.[129,130] investigated the orientation of actin labeled with rhodamine phalloidin relative to the cell membrane. This study was achieved by clamping red cell ghosts and using polarization imaging to measure the ratio of the parallel to perpendicular intensity to determine that the actin filaments have the same membrane tangent orientation under significantly different axial and radial stress conditions. Rosenberg et al.[131] describe, among other studies, single molecule polarization experiments applied to actin filaments to determine their mobility and orientation. They also include a discussion of simple molecule polarization imaging considerations. Corrie et al.[132] used an ingenious method to eliminate local probe mobility from a covalently attached probe; specifically, they designed a bifunctional iodoacetamidorhodamine label and attached it to the myosin regulatory light chain genetically modified to encode four different pairs of solvent accessible cysteines. Fluorescent labeling in this manner provides a significant advantage over attachment with only a single covalent bond, namely the reduction or complete elimination of local motion of the probe. They exploited the lack of local probe motion to determine the twist and tilt of the myosin light chain with respect to actin filaments within individual muscle cells. Of course, caution must be used whenever such chemical substitutions to the xanthene rings of rhodamine or fluorescein based compounds are made, since this alteration may alter the probe's excitation polarization spectrum (Figure 7 and M. Anson personal communication).

Sase et al.[133] used fluorescently labeled actin filaments to follow the rotation of the actin with time over many rotations of the fiber. From these experiments, they determined that the actin filament was translated a distance of around 1 μm per rotation and thus the rotation of the filament does not follow the pitch of the actin helix. Polarization imaging has also been applied to flow cytometry by Eirsert et al.,[111] who determined a polarization histogram for fluorescently labeled latex spheres, for DNA in the presence of ethidium bromide, and for algae. Campagnola et al.[134] combined second harmonic generation (SHG) using multiphoton imaging with polarization imaging to investigate the orientation of endogenous protein polymer structures within scales of the black tetra fish. They found that, unlike SHG from a frequency doubling crystal (e.g., BBO), whose SHG emission is polarized orthogonal to the excitation polarization, the SHG emission was oriented parallel to the excitation polarization. An excellent review on the use of polarized fluorescence microscopy to investigate the actin-myosin system and *in vitro* motility assays has been presented by Yan and Marriott.[114]

Pu et al.[135] used time-resolved and steady state fluorescence techniques including decay anisotropy and polarization imaging to compare cancerous and noncancerous tissue types using intrinsic and extrinsic fluorophores. From the near-infrared spectral wing of the intrinsic fluorophores of the tissue, they show that the cancerous tissue has a higher fluorescence intensity and time-dependent polarization.[135] The same group also used indocyanine green coupled to a short peptide consisting of the somatostatin receptor ligand as a contrast agent and compared the initial anisotropy and the time-resolved anisotropy of stained cancerous and normal tissue.[136]

Harms et al.[137] used polarization imaging to measure the rotational and lateral mobility of fluorescently labeled lipids in single molecule experiments.[137] They employed a Wollaston prism in the emission path to spatially separate the different polarizations and image with a CCD whereas most implementations of polarization imaging use two PMTs with a polarizer in front of each. Blackman et al.[138] used polarized confocal fluorescence microscopy to determine the orientation of the major integral membrane protein of erythrocytes, band 3, extrinsically labeled with eosin-5. Their results were consistent with a distribution of orientations of the eosin normal to the membrane. Rocheleau et al.[139] used two GFP modified major histocompatibility complex molecules, one within the amino acid sequence of the protein and one at the C-terminus, to analyze the synthesis and assembly of the complex. In general, fluorescent proteins are not very suitable as probes for polarization studies due to their relatively short lifetime (1−4 ns) compared to their rotational correlation time. However, Rocheleau et al.[139] used the depolarization of the fluorescence due to the homo-FRET between adjacent GFP molecules to determine that, prior to peptide loading, the GFP constructs were aggregated in the ER but that, in the presence of peptide loading, the molecular aggregates dispersed. While the critical Förster distance for GFP-GFP is not as large as that for other fluorescent protein pairs (~4.65 nm),[140] energy transfer is still possible and will result in a smaller polarization upon molecular aggregation.

Suzuki et al.[141] used a variety of fluorophores, including RH292, calcium green, and SNARF to investigate membrane reorganization in sea urchin eggs. They employed a "multiview" imaging technique where images were acquired in a 2×2 manner with the images separated spectrally and based on polarization. Several groups have also extended the use of steady state polarization imaging to implement time-resolved methods, e.g. refs 142−148, but a detailed discussion of this topic is beyond the scope of this review.

8. Fluorescent Probes

In 1856, while attempting to synthesize quinine, William Henry Perkin synthesized the dye mauve, a derivative of coal tar with an aniline base. Up until this time, virtually all pigments were derived from natural sources. Other chemists had synthesized some pigments, but Perkins realized the commercial potential of the compounds and so is considered the father of the synthetic dye industry.[149]

8.1. Fluorescein

Fluorescein, perhaps the most popular fluorescent probe in the world and undoubtedly the most popular probe used in clinical chemistry/high throughput screening, was first synthesized, and christened, by Adolph Baeyer in 1871.[150] Although the compound he synthesized was more properly named "spiro[isobenzofuran-1(3H),9′-[9H]xanthen]-3-one, 3′,6′-dihydroxy", we can all be grateful that Baeyer chose a common name in recognition of the fluorescence properties ("fluo")

and also of the synthetic route ("rescein"), which involved reaction of phthalic anhydride with resorcinol or resorcin.

It is interesting to speculate on why fluorescein continues to be such a dominant fluorophore. Some of the reasons for its popularity include the following: (1) it is relatively inexpensive, (2) it is not patented (as opposed to most new probes, such as the Alexa series), and (3) it is adaptable to several different chemistries to allow conjugation with functional groups such as amines or sulfhydryls. Given the widespread use of fluorescein in immunoassays and high throughput screening, we should consider some of the salient aspects of its photophysics. Fluorescein does suffer from photodegradation, especially in the presence of oxygen,[151] and its spectral properties (including quantum yield, extinction coefficient, and fluorescence lifetime) vary as a function of pH.[151−153] It is thus important for an investigator using fluorescein as part of an assay (especially a polarization assay) to appreciate the effect of pH on the spectral properties.

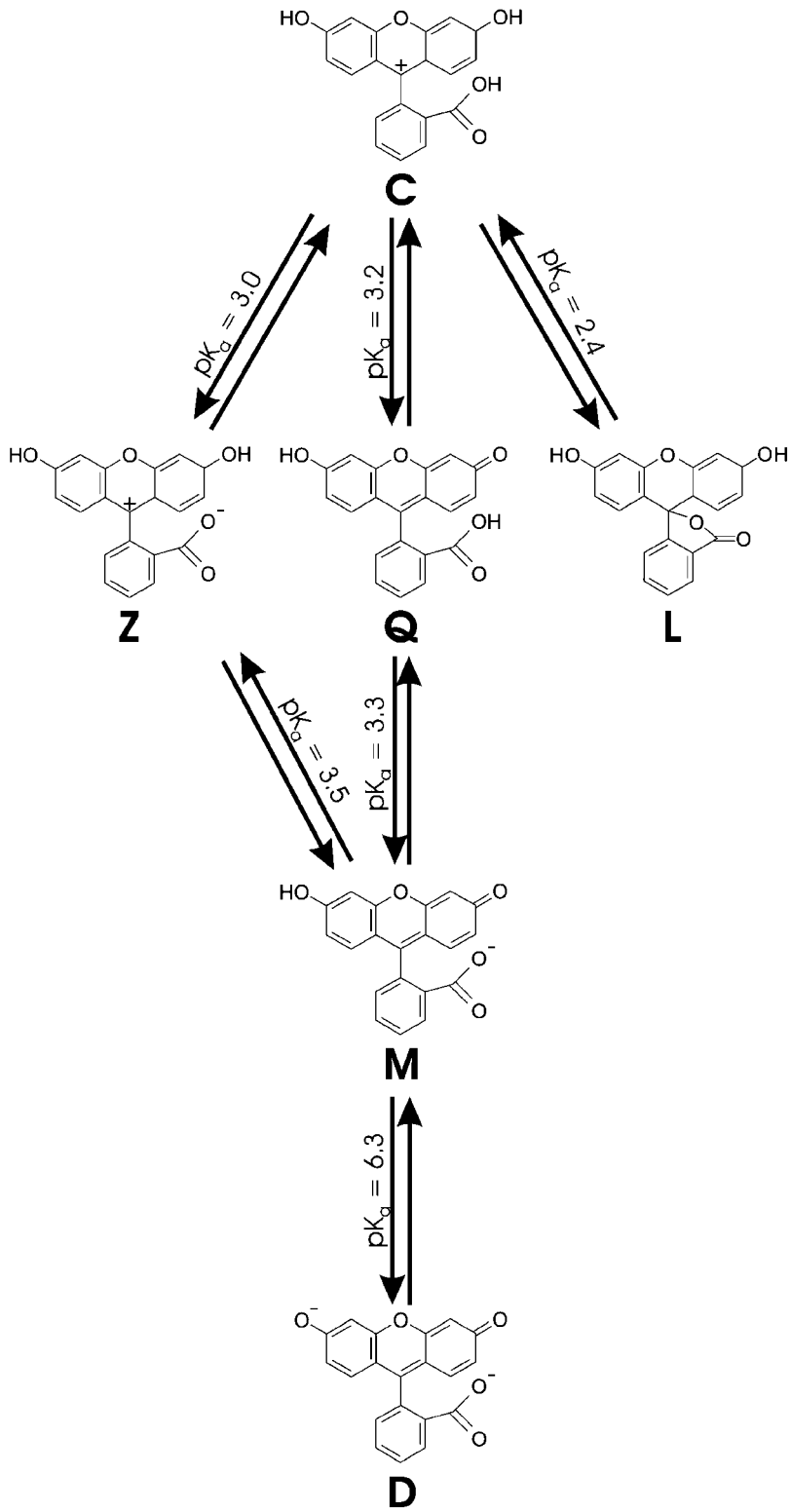

Figure 16. Chemical structures of the various prototropic forms of fluorescein showing the pK_a of each transition: C, cation; Z, zwitterion; Q, quinoid; L, lactone; M, monoanion; D, dianion.

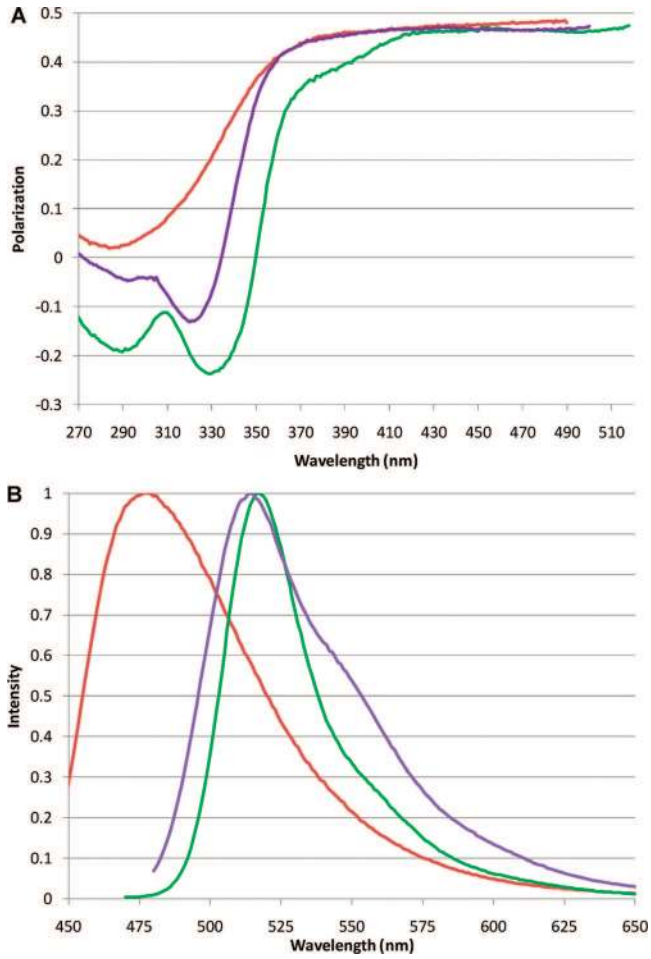

Figure 17. (A) Excitation polarization spectra and (B) emission spectra of fluorescein in 0.01 M NaOH (green), 0.01 M HCl (red), and 0.01 M MES buffer pH 6 (purple) at 2 °C in glycerol.

Fluorescein may be present in one or more prototropic forms (Figure 16). At alkaline pH, fluorescein is predominantly in the dianion form. As the pH drops, the hydroxyl group becomes protonated while the carboxylic acid remains deprotonated, i.e., the monoanion form; as the pH drops further, it undertakes one of several forms, either a quinoid, lactone, or zwitterion. The lactone and zwitterion forms are nonfluorescent, with the lactone form being the predominant species at neutral pH. The absorption and emission maxima of fluorescein at alkaline pH are approximately 490 and 520 nm, with the fluorescence lifetime being 4.05 ns. The limiting polarization at 490 nm excitation is high, ~0.48. Different prototropic forms of fluorescein have different spectral properties, including absorption and emission spectra as well as excitation polarization spectra. Some of these differences are illustrated in Figure 17.

8.2. FITC

The most commonly used reactive form of fluorescein is fluorescein isothiocyanate (FITC), which is commonly available as two isoforms, I and II (Figure 18). FITC reacts with primary amines and is often used to label lysine residues in proteins (note: for efficient lysine labeling, the pH should be 8 or higher to ensure that a significant fraction of the ε-amino groups are deprotonated). Albert Coons is sometimes given credit for introducing FITC to immunochemistry, but he and his colleagues actually synthesized fluorescein isocyanate[154] (although Coons, of course, deserves considerable

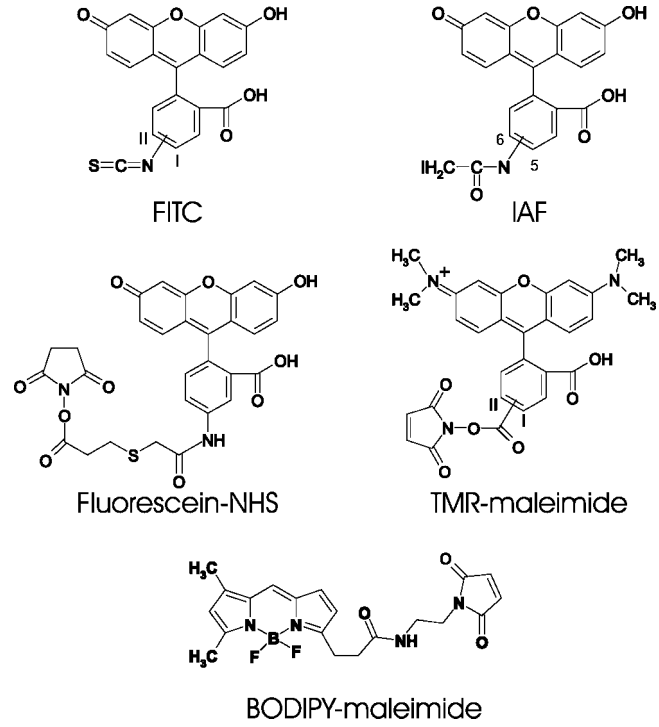

Figure 18. Chemical structures of commonly used reactive probes: fluorescein-isothiocyanate (FITC), iodoacetamido-fluorescein (IAF), fluorescein *N*- hydroxysuccinimidyl ester (fluorescein-NHS), tetramethylrhodamine-maleimide (TMR-maleimide), and BODIPY-maleimide.

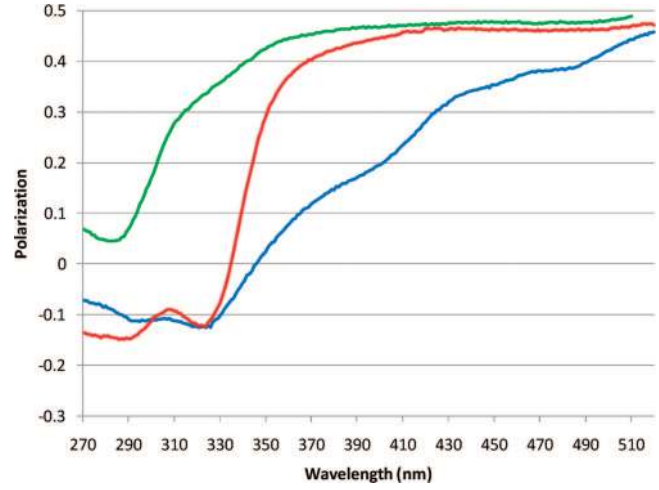

Figure 19. Excitation polarization spectra of fluorescein-isothiocyanate (FITC) (blue), iodoacetamido-fluorescein (IAF) (red), and Green Fluorescent Protein (GFP) (green) in glycerol at 2 °C.

credit for having the idea to link antibodies with fluorophores). Riggs et al.[155] first reported FITC, in 1958, which they synthesized to circumvent problems inherent in the isocyanate derivative, including the difficulty of its synthesis and its instability. Other reactive species of fluorescein include iodoacetamidofluorescein, which reacts with sulfhydryl groups (hence, it is used to target cysteine residues in proteins) and *N*-hydroxysuccinimidyl fluorescein, which also reacts with primary amines (Figure 18). The distinct chemical groups involved in the different linkage chemistries may also alter the excitation polarization spectra of probes, as illustrated for several fluorescein derivatives in Figure 19. We note that the use of one probe isomer versus another can actually have significant effects on the photophysical properties under investigation. For example, Blackman et

al.[156] found that the 5′ and 6′ isomers of iodoacetamidorhodamine derivatives of peptides had different propensities to form ground-state excimers when linked to the same peptides (we note that xanthene dyes have a propensity to form ground state dimers when in proximity; these dimers are often nonfluorescent and have altered absorption properties[157−161]). Another subtlety of fluorescent isomers was reported by Oiwa et al.,[162] who found that Cy3 dyes linked to either the 2′ or 3′ ribose ATP exhibited very different intensities, lifetimes, and rotational properties.

8.3. Other Probes

Although fluorescein has tended to dominate applications of fluorescence polarization, there are certainly many other reactive probes described in the literature. In the 1960s and 1970s, Gregorio Weber synthesized and characterized several probes which are still popular, including IAEDANS[163] (the first sulfhydryl reactive probe), pyrenebutyrate[164] (which has a long lifetime and which was introduced to study very large proteins), and PRODAN[165] (designed to have a very large dipole moment in the excited state and hence useful to monitor the polarity of the probe environment—other probes in this family include LAURDAN[166] and DANCA[167]). The sulfhydryl reactive derivative of PRODAN is Acrylodan.[168] Other probes commonly used for polarization assays include BODIPY and tetramethylrhodamine (TMR) (Figure 18). The commercialization of fluorescence probes began in earnest with the advent of Molecular Probes by Richard Haugland in the late 1970s. Haugland had been a postdoctoral fellow with Lubert Stryer and had synthesized fluorescent molecules designed to test aspects of FRET theory.[169,170] Molecular Probes provided a huge impetus to the fluorescence field by eventually making thousands of fluorescent molecules available to researchers. Haugland and his associates also introduced new classes of highly photostable dyes, such as the Alexa series,[171] which became especially important in applications involving fluorescence microscopy. In recent years, numerous other companies began to sell specialized fluorescent molecules, a testament to the commercial importance of this area. A review of fluorescent probes used in chemical biology was written by Lavis and Raines,[172] who present an excellent overview of fluorescent molecules organized according to their chemical families as well as their useful excitation and emission wavelength ranges. Figure 2 in their article presents a marvelous pictorial summary of the brightness (i.e., the product of a fluorophore's extinction coeffient and its quantum yield) and absorption and emission properties of many important fluorescent molecules.

8.4. The Green (Fluorescent Protein) Revolution

An excellent overview of the "Fluorescent Toolbox" available to cell biologists was presented by Roger Tsien and his colleagues in 2006.[173] Foremost in this toolbox are the fluorescent proteins. Osamu Shimomura (who was interested in the bioluminescence of the aequorin jellyfish) and his colleagues[174] first described the purification and spectroscopic properties of the so-called "green fluorescent protein" (Woody Hastings actually coined this name[175]), and the rest—as they say—is history. The family of fluorescent proteins, from phyla including *Cnidaria* and *Arhtropoda* as well as from other several species,[176] and the numerous mutations which give rise to a multitude of absorption and fluorescent properties[177] have become extremely important in cell studies including evaluation of cellular processes and metabolism. These proteins are also becoming increasingly popular as biosensors, both *in vitro* and in living cells. The excitation polarization spectrum for GFP is shown in Figure 19.

9. Fluorescence Instrumentation

The steady state polarization fluorimeter (we note that the custom of calling steady-state instruments "fluorimeters" and time-resolved instruments "fluorometers" goes back to Gaviola,[178] who called his original lifetime instrument "Ein Fluorometer") may take several forms depending on the sample platform, e.g. cuvette, multiwell plate, etc. The basic principle of the measurement remains the same though. An excitation light source excites the sample with parallel polarized light. The intensity of the emission light is then collected through parallel and perpendicular polarizers, and the polarization is determined (this approach of rotating the emission polarizer is most traditional, but variations exist wherein the emission polarizer is fixed and the excitation polarizer is rotated).

The excitation source of the fluorimeter is typically a Xe or Hg arc lamp, which is spectrally filtered using either a monochromator or a bandpass (or interference) excitation filter. Alternatively, a newer generation light source may be used such as a laser, laser diode, or LED. The advantage of a lamp source is that the excitation wavelength can be continuously varied as required, whereas, for a laser, laser diode, or LED, the excitation wavelength either is restricted to discrete values or is only tunable over a limited range (see, however, the discussion in section 9.1 on two-photon lasers). However, these light sources have a much higher intensity at their particular operating wavelength than a lamp, and the laser sources can produce intrinsically partially or completely polarized light.

Following the excitation source, but prior to the sample, is the excitation polarizer. The absorbing types of polarizer are commonly thin films, e.g., Polaroid type-H sheets based on stretched polyvinyl alcohol impregnated with iodine. These types of polarizers are inexpensive but can be damaged by high light intensities and also have poor transmission at UV wavelengths. Alternatively, the polarizer may be made from a pair of birefringent prisms, typically calcite. The relative orientation of the ordinary and extraordinary axes results in one polarization of light being transmitted and the other being either reflected at a large angle or spatially separated from the transmitted beam. Calcite polarizers are more expensive than sheet polarizers but have much higher extinction ratios for the parallel versus perpendicular components and can sustain prolonged high intensity illumination without damage; they also have a greater transmission in the ultraviolet (see, for example, ref 21 for a comparison of several polarizers). Following the sample is the emission polarizer, which is rotatable through 90° to allow determination of the parallel and perpendicular light intensities. The emission polarizer is generally of the same type as the excitation polarizer. The emission must then be spectrally filtered to remove scattered excitation light. This task can be accomplished using either a filter (e.g., long-pass or interference filter) or a monochromator. The presence of scattered light will increase the polarization because it will be mostly vertically polarized. However, in the absence of scattered light—either Rayleigh, Raman, or parasitic light—

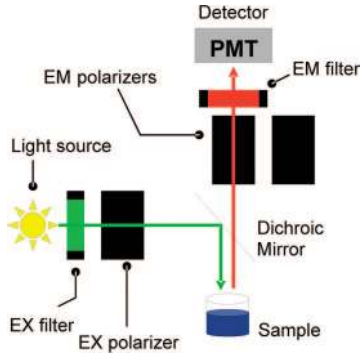

Figure 20. Diagram of a typical fluorescence polarization plate reader, showing the light source, sample, detector, and excitation and emission filters and polarizers. Image courtesy of Xavier Amouretti and Bio Tek Instruments, Inc.

sample turbidity will actually decrease the observed polarization due to multiple scattering of the emission. For example, for the case of dansyl-labeled bovine serum albumin, upon 366 nm excitation, the observed polarization will decrease from 0.306 to 0.250 upon addition of 0.7% glycogen, which raises the optical density of the solution from 0.02 to 0.33.[179] If turbidity is inherent in the sample, this effect can be mitigated somewhat by using shorter optical path lengths. We should also note that blank subtraction procedures will not correct for depolarization of the emission by scattering. A sketch of the light paths for a plate reader instrument is shown in Figure 20. For more detailed general discussions of fluorescence instrumentation, see refs 17, 18, and 180.

9.1. Two-Photon Excitation

The vast majority of polarization measurements are realized using one-photon excitation (although, see Tirri et al.[109]). In recent years, two-photon excitation has become very important in fluorescence microscopy applications. With two-photon excitation, a very high local photon density (usually at near-infrared wavelengths) is achieved at the focal spot of the objective and fluorophores can experience two-photon absorption, essentially the simultaneous absorption of two near-infrared photons, resulting in excitation of the fluorophore to the same first excited singlet state, normally achieved via a one-photon process (for discussions of multiphoton methods and of two-photon cross sections of various fluorophores, see [181−184]). Two-photon excitation is increasingly popular due to (1) its inherent optical sectioning, i.e., the confocal aspect, (2) the fact that, for work with living cells, the phototoxicity of the out of focus near-infrared illumination is generally much lower than that of one-photon excitation, and (3) the ability to eliminate Rayleigh or Raman scatter from the observed emission. This last point is, of course, one of the great advantages recommending two-photon excitation for fluorescence polarization measurements, since, as mentioned earlier, scattered light, due to Rayleigh or Raman scattering, can significantly increase the observed polarization. Another advantage of two-photon excitation is that the limiting polarization is higher than that for one-photon excitation, reaching levels of 0.67.[21] The reason for this higher limit is that every photon absorbed carries out a photoselection process so that the probability of absorption depends upon $\cos^4 \theta$. Some of the advantages of the two-photon format in bioassays in general have been presented by Hanninen et al.,[185] and in fluorescence polarization measurements in particular by Tirri et al.[109] The disadvantages of two-photon excitation would be as follows: (1) since the two-photon absorption cross sections of fluorophores are very broad, other potential, unwanted fluorophores in a mixture may be excited in addition to the target molecule, and (2) it is fantastically expensive compared to one-photon light sources!

10. Conclusions

As the reader should now appreciate, fluorescence polarization/anisotropy offers significant advantages for some applications in clinical chemistry and bioassays and its use in biotechnology and drug discovery will no doubt continue to expand. In this review we have endeavored to provide a theoretical framework and also to discuss certain practical aspects of the method to assist those who seek to rationally design new fluorescence polarization assays. We have also sought to provide some perspective on the history of these methods so that those who are new to the field can appreciate the work of their predecessors and hopefully not have to "reinvent the wheel" in their own research.

11. Acknowledgments

We wish to thank Mick Jolley for valuable discussions and insight on the development of the Abbott TDx instrument. We also thank Xavier Amouretti and Bio Tek Instruments, Inc. for providing Figure 20 and Otto Baumann from the University of Potsdam and Eva Simbürger and Oliver Holub from Carl Zeiss MicroImaging GmbH for Figure 15E and F. This work was supported in part by a grant from the National Institutes of Health (RO1GM076665) and by a grant from Allergan, Inc.

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CR900267P