Fluorescence Polarization: Past, Present and Future

David M. Jameson* and John C. Croney

Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, 1960 East-West Rd. HI 96822; Honolulu, USA

Abstract: Fluorescence polarization was first observed in 1920 and during the next few decades the theoretical foundations of the phenomenon were clearly established. In the last two decades of the 20th century, fluorescence polarization became one of the most prevalent methods used in clinical and biomedical sciences. In this article we review the history of fluorescence polarization, its theoretical foundations and some of the more important practical developments, which helped to popularize the method. We also discuss important, but often misunderstood, practical considerations including the wavelength dependence of the limiting polarization and the effect of energy transfer on polarization. The present state of fluorescence polarization, both in pure research as well as in the applied biosciences is also reviewed. Finally, we speculate on possible future developments in the field, such as the use of multi-photon techniques.

INTRODUCTION

The essential aspect of fluorescence polarization, which makes it useful in the biomedical field, is that it provides information on the rotational mobility of a fluorescent molecule. Since the mobility of a fluorescent molecule will change if it binds to another molecule, one can utilize fluorescence polarization to study and quantify biomolecular interactions. Consequently, fluorescence polarization has been used for many years to study biochemical systems, such as protein-protein and protein-ligand interactions, membrane dynamics and, in recent years, protein-DNA and DNA-DNA interactions. In the last two decades, fluorescence polarization methods have also been increasingly used in medical diagnostics and high-throughput screening. As happens when any scientific discipline reaches a certain level of maturity, the newest practitioners may lose sight of the most basic principles underlying the method and may not always appreciate the pioneers who toiled to establish the theoretical and experimental foundations. One of the motivations behind this article is to remind readers of salient aspects of the historical development of the method along with basic principles and some potential pitfalls. Some possible future directions will also be discussed, but experience shows that the most interesting future developments are those that we never imagine.

HISTORICAL OVERVIEW

In 1920, F. Weigert discovered that the fluorescence from solutions of dyes was polarized [1]. Specifically, he looked at solutions of fluorescein, eosin, rhodamine and other dyes and noted the effect of temperature and viscosity on the observed polarization. Weigert discovered that polarization increased with the size of the dye molecule and the viscosity of the solvent, yet decreased as the temperature increased. He recognized that all of these considerations meant 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 Levschin in 1923 [2], 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 polarization in glycerol, whereas other dyes gave similar polarizations regardless of the solvent's viscosity. 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. In 1925 - 1926, Francis Perrin (son of the famous French physicist, Jean Perrin), published several important papers describing a quantitative theory of fluorescence polarization [3-5], including what is now considered his classic paper [5] containing most of the essential information that we use to this day (for a really excellent discussion of Jean and Francis Perrin's outstanding contributions to fluorescence the reader is directed to Berberan-Santos's article) [6].

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 in Cambridge in the mid-1940's. Weber was strongly influenced by Perrin's article and in later years wrote [7].

"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

*Address correspondence to this author at the Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, 1960 East-West Rd. HI 96822; Honolulu, USA; Phone: (808) 956-5034; Fax: (808) 956-9952; Email: djameson@hawaii.edu

1386-2073/03 $41.00+.00

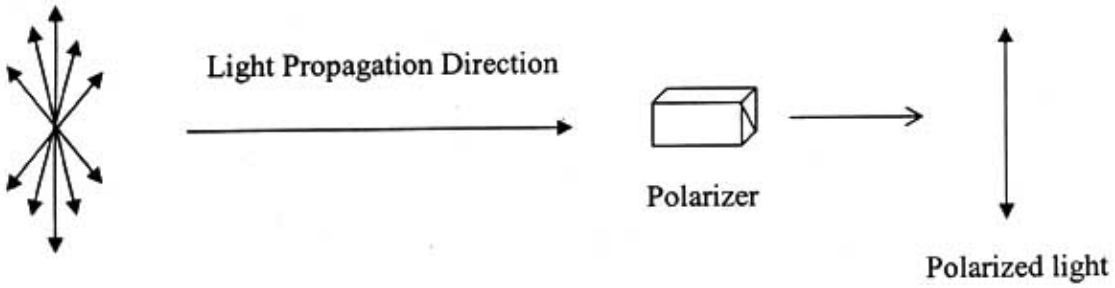

Fig. (1). Diagram showing the orientation of electric vectors in natural light and the isolation of vertical polarized light through a polarizer.

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's early studies concerned flavins and flavoprotein systems - intrinsic protein fluorescence had not yet been discovered. In the early 1950's Weber synthesized the fluorescent probe dimethylaminonaphthalene sulfonyl chloride - dansyl chloride – which could be covalently linked to proteins. Weber's subsequent theoretical and experimental work – which extended Perrin's earlier contributions and also developed what became modern instrumentation - brought fluorescence polarization to the attention of the biochemical community, and so ushered in a new scientific discipline – quantitative biological fluorescence. In addition to designing numerous probes for specific protein and membrane applications, Weber also carried out the first quantitative work on intrinsic protein fluorescence. For a review of Gregorio Weber's seminal contributions to fluorescence see [8].

THEORETICAL BASIS

Light can be considered as oscillations of an electromagnetic field perpendicular to the direction of propagation, as shown in figure **1**. Although both electric and magnetic components are present, we shall be concerned only with the electric field. A polarizer[1] is an optical component, which selectively transmits one direction of the electric field oscillation, as illustrated in figure **1**. The most common polarizers used today are (1) dichroic devices, which operate by effectively absorbing one plane of polarization (e.g., Polaroid type-H sheets based on stretched polyvinyl alcohol impregnated with iodine) and (2) double refracting calcite ($CaCO_3$) crystal polarizers - which differentially disperse the two planes of polarization (examples of this class of polarizers are Nicol polarizers, Wollaston prisms and Glan-type polarizers such as the Glan-Foucault, Glan-Thompson and Glan-Taylor polarizers)[2].

In a typical fluorescence experiment, the emission, excited by vertically polarized light (isolated by appropriate wavelength selection devices such as filters or monocromators) is observed at right angles to the excitation (to minimize scattered light in the observation direction), and the polarization of the emission can then be determined by observation through both vertically and horizontally oriented polarizers. In this case, vertical and horizontal refer to the direction of the vertical laboratory axis and are also called the parallel and perpendicular orientations, respectively. This arrangement is illustrated in figure **2**. The polarization is then defined as:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities observed in the parallel and perpendicular directions, respectively[3]. One notes that negative polarizations are possible if the perpendicular intensity exceeds the parallel intensity – such a situation can easily occur and will be discussed later. As stated earlier, the correct quantitative relation between the observed polarization and parameters such as the excited state lifetime, the size of the fluorophore and the viscosity of the solution was enunciated by F. Perrin in 1926 [5]. In this classic 1926 article, the equation, now known as the Perrin equation, first appears as:

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

where P is the polarization, P_0 is the limiting polarization (in the absence of rotation), R is the universal gas constant, T is the absolute temperature, V is the molar volume of the

[1] Polarizers have, in fact, been in use for a very long time - the Vikings used a "sunstone" (now thought to have been composed of the mineral cordierite, a natural polarizing material) to observe the location of the sun on foggy or overcast days. Since scattered sunlight is highly polarized compared to light coming along the direction to the sun, the distribution of the sky's brightness could be observed through the sunstone and hence the sun's position could be localized and, if the time of day were known, the compass directions.

[2] A comparison of the efficiencies of several types of polarizers was given by Jameson et al. (*9*).

[3] To properly determine polarizations, any bias in the detector arm (either due to monochomator or PMT response) must be taken into account, an operation usually accomplished by measuring the parallel and perpendicular polarized intensities of the emission while exciting with perpendicularly polarized light, i.e., in a direction which is orthogonal to both emission components (due to the right angle observation). In this symmetrical excitation arrangement, the two emission polarization components should be equal, and deviation from equality can be ascribed to instrumental bias and corrected. This concept was, of course, recognized at the very beginnings of polarization research. The correction is now usually referred to as the "G-factor" after the nomenclature of Azumi and McGlynn (*10*) who used this term to refer to the bias of the grating in the emission monochromator.

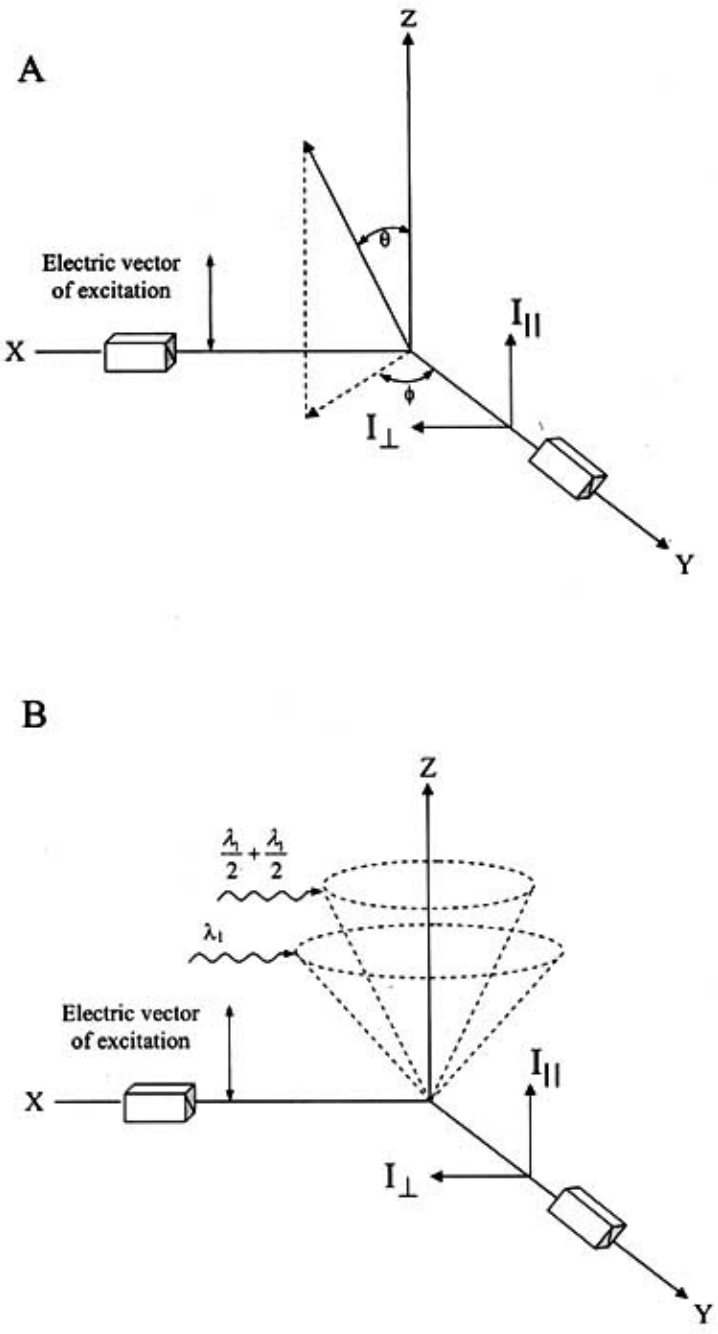

Fig. (2). A. Diagram depicting (a) photoselection of an absorption dipole, making angles θ with the Z –axis (in the XZ plane) and ϕ with the Y-axis (in the XY plane) by vertically polarized light and (b) observation along the Y-axis of the vertical (parallel) and horizontal (perpendicular) components of the emission through a polarizer. **B**. Depiction of the photoselection process for one-and two-photon excitation.

rotating unit, η is the solvent viscosity and τ is the excited state lifetime. This equation is usually now written as:

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

which is often rearranged as:

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

where ρ is the Debye rotational relaxation time (this equation is sometimes referred to as the Perrin-Weber

equation). The rotational relaxation time for a spherical molecule, ρ_o, is given by:

$$\rho_o = \frac{3\eta V}{RT}$$

We should comment here on the term "rotational correlation time", often denoted as τ_c. In fact, $\rho = 3\tau_c$, a fact that stems from the original definitions of these terms. As pointed out previously [11], the rotational relaxation time was originally defined by Peter Debye in relation to dielectric dispersion in which the relevant orientational distribution is a function of $\cos\theta$, where θ is the angle between the direction of the electric field and the molecular dipole axis (figure **2A**). Perrin used this approach of Debye when he derived the characteristic time for molecular rotation of spheres. Much later, Bloch derived equations for computing the decay of nuclear polarizations and used a slightly different approach, which yielded a characteristic time which was one third the Debye rotational relaxation time, i.e., the correlation time.

In the literature, one often sees the function known as anisotropy (r), which is defined as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

The Perrin equation can then be expressed as:

$$\frac{r}{r_o} = 1 + \frac{\tau}{\tau_c}$$

where r_0 is the limiting anisotropy and τ_c is the rotational correlation time as discussed above. The simplified form of this equation, compared to the original Perrin equation, has attracted users. We wish to stress that the information content of the various terms, polarization/anisotropy or relaxation time/correlation time, is identical and the most important consideration is to clearly specify which terms are being used. It is interesting to note that the diagnostic field, in general, uses the polarization function and moreover that the unit known as "millipolarization" or "mP" is almost universally utilized – mP is simply the polarization times 1000 (e.g., a polarization of 0.200 becomes 200mPs).

APPLICATIONS TO LIGAND BINDING

The first application of fluorescence polarization to monitor the binding of small molecules to proteins was carried out by D. Laurence in 1952 using Gregorio Weber's instrumentation in Cambridge [12]. Specifically, Laurence studied the binding of numerous dyes, including fluorescein, eosin, acridine and others, to bovine serum albumin, and used the polarization data to estimate the binding constants. Dandliker and his coworkers later applied these principles explicitly to study antibody-antigen [13] and hormone-binding site interactions [14]. One of the first instruments designed specifically for clinical chemistry applications of fluorescence polarization was described by Spencer *et al.* [15]. Among the first commercial instruments designed for this purpose was, of course, the Abbott TD_x [16-18]. For an excellent review of this field see [19] and, of course, the other articles in this issue! Binding stoichiometries and dissociation constants can be readily determined from polarization data and many such studies have appeared (for a review see[11]). Perhaps the two most important points to keep in mind in such studies are (1) how do the polarizations of the individual components – *i.e.*, free and bound probe – contribute to the observed polarization and (2) how should any change in quantum yield upon binding be handled. Weber anticipated these questions in his original theory article [20] and showed that the additivity of polarizations is given by:

$$\left(\frac{1}{P_{robs}} - \frac{1}{3}\right)^{-1} = \Sigma f_i \left(\frac{1}{P_i} - \frac{1}{3}\right)^{-1}$$

where P_{obs} is the actual polarization observed arising from i-components, f_i represents the fractional contribution of the ith component to the total emission intensity and P_i is the polarization of the ith component. This additivity principle was later expressed in terms of anisotropy (r) by Jablonski [21] as: $r_{obs} = \Sigma f_i r_i$. Although the anisotropy formulation is simpler in appearance, the information content of the two approaches is identical and given the present day computer-assisted data analysis the difference is moot. Clearly, if the quantum yield of the fluorophore changes upon binding, the fractional intensity terms in Weber's additivity equation will alter. Although many probes (such as fluorescein) do not significantly alter their quantum yield upon interaction with proteins, one should not take this fact for granted and would be well advised to check. If the quantum yield does in fact change, one can readily correct the fitting equation to take the change in yield 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) is [22]:

$$X = \frac{r - r_f}{r_b - r_f + (g - 1)(r_b - r)}$$

WAVELENGTH DEPENDENCE OF POLARIZATION

As derived elsewhere [23-25], the limits for polarization from a solution of randomly oriented fluorophores are +1/2 and –1/3 depending on whether the absorption and emission dipoles are collinear (+1/2) or orthogonal (-1/3). The expression relating the limiting polarization (P_0) to the angle (ϕ) between the absorption and emission dipoles is:

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

Normally, emission results from excitation near the fluorophore's absorption maximum and in this case the absorption and emission dipoles are often close to co-linear. Hence the approximation of +0.5 for P_0 will not usually lead to significant errors. In recent years, though, the advent of laser sources and laser diodes, coupled with a plethora of new dyes with widely diverse absorption characteristics, has meant that polarization measurements are not always excited into the final absorption band, which can result in a lower P_0 value and hence a lower polarization than expected. The fluorescence practitioner should be very aware of the

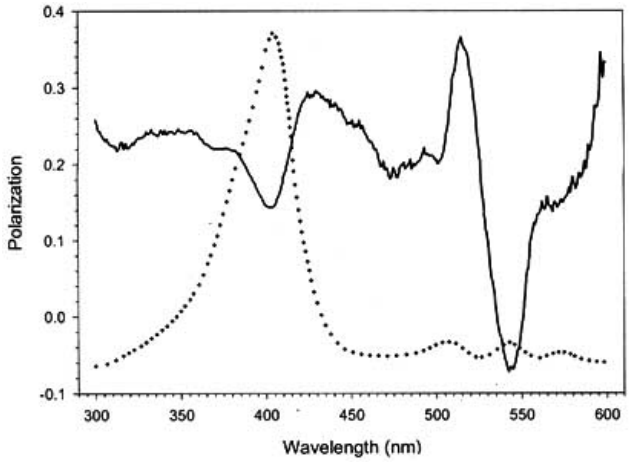

Fig. (3). Absorption spectrum (dotted line) and excitation polarization spectrum (solid line) of protoporphyrin IX in glycerol –20C. Emission was observed through a 631nm (10nm FWHM) interference filter.

fluorophore's P_0 value at the excitation and emission wavelength utilized. The excitation polarization spectrum of a fluorophore gives the limiting or P_0 value (a given emission wavelength range) as a function of the excitation wavelength. An example of such a polarization spectrum is shown in figure **3** for protoporphyrin IX in glycerol at –20 °C. (unpublished data of G.D. Reinhart and D.M. Jameson). As is evident from this spectrum, the observed polarization will depend dramatically upon the exciting wavelength and furthermore the limiting value never approaches +0.5. This example is more complex than many of the fluorophores in common use (for excitation polarization spectra of other fluorophores see [50] or [25]) but illustrates the point. An even more insidious case occurs when the polarization varies as a function of the emission wavelength. In the case of most fluorophores, polarization is constant across the emission spectrum, which is why cut-on filters can be used to collect the total emission and still maintain the highest possible polarization value. Some cases exist, however, in which the polarization varies with both excitation and emission wavelength – an example is chrysene, shown in figure **4**. In fact, pyrene, a commonly used probe, has intrinsic polarization properties similar to chrysene and hence one must choose both the excitation and emission wavelengths carefully to maximize its polarization value. Finally, one other case of interest is that of symmetrical molecules with degenerate emission states. In such cases, polarized excitation into the final absorption band selects molecules with their absorption dipoles aligned with the polarization direction of the exciting light, but then the degeneracy of the excited states allows for the emission to occur from differently oriented dipoles. Triphenylene, for example, has three-fold symmetry and emission will come equally from three directions oriented at 120^o with respect to one another. It is a useful exercise for the reader to demonstrate that such three-fold symmetry results in a limiting polarization of +1/7 [26].

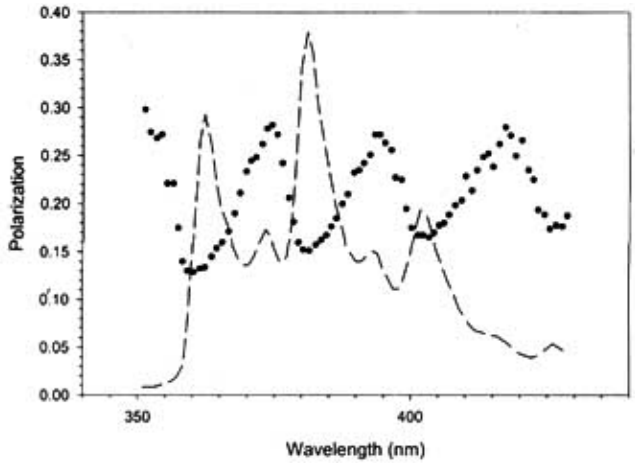

Fig. (4). Emission spectrum (dashed line) and emission polarization spectrum (dotted line) of chyrsene in glycerol at –60C. Excitation was at 320nm.

PRACTICAL ASPECTS AND INSTRUMENTATION

The first detailed characterization of a modern polarization instrument was that by Weber in 1956 [27] entitled "Photoelectric method for the measurement of the polarization of the fluorescence of solutions". Readers with a serious and sustaining interest in polarization instrumentation would do well to study this classic paper since it discusses, in quantitative terms, the most important instrumental and optical considerations that effect the accuracy of the polarization measurement, including the precise effect of misalignment of excitation or emission polarizers and the effect of the numerical aperture of the lenses utilized. The numerical aperture issue is important if one is concerned with the "accuracy" of the polarization determination. Most clinical and screening applications rely on relative changes in polarization, i.e., between free and bound ligands. Aperture effects are also clearly relevant to microscopy applications - specifically, the larger the numerical aperture of the lenses focusing the excitation and collecting the emitted light (i.e., the shorter the focal length and hence the larger the cone of collected light) the lower will be the measured polarization compared to the true polarization. The influence of numerical aperture on measured polarizations has been treated explicitly by numerous groups including [27-30]. In normal, *i.e.*, non-microscopic, research quality spectrofluorimeters (such as from SLM or ISS), the deviation of measured polarization from its true value is only a few percent – due to collection optics that typically subtend about 15º – but it is interesting to note that Weber's original instrument had less error since he used lenses which subtended only ~2.5º. The trade-off, of course, is larger light collection efficiency versus more accurate polarization values. In fluorescence microscopes, the magnitude of the effect is almost negligible for numerical apertures below 0.5 (typical for a 20X objective) but can lead to an observed polarization ~20% below the true value for a numerical aperture of 1.3 (typical for a 100X objective)[30].

Although Weber's early instruments utilized a "T-format" optical arrangement, i.e. two detector arms at right angles to the excitation direction, one with a polarizer fixed parallel and the other with a polarizer fixed perpendicular, it is more common now to use instruments with an "L-format" arrangement wherein one emission polarization arm is used and the polarizer is rotated between the parallel and perpendicular positions. Weber's original T-format design had the advantage that only the excitation polarizer had to be rotated (between the parallel and perpendicular positions), hence the measurement was faster and there were fewer movable parts which could, through use, slip out of alignment. Modern instruments, however, with automation and computer control, render these advantages less significant. Additionally, some instruments originally designed for rapid and simple clinical applications use liquid crystals to rotate the polarization plane and thus avoid some problems attendant to moving parts [18]. Other more recent commercial instrument innovations include multiwell plate polarization readers such as the Analyst^{TM} or Acquest^{TM} family of instruments from Molecular Devices, the Fusion^{TM} Universal Microplate Analyzer and the Wallac Victor^{2}V multilabel reader from Perkin-Elmer, the Polarion system from Tecan, the POLARstar Optima from BMG Labtechnologies and the FARCyte system from Amersham Biosciences (this list is not meant to be exhaustive). At the time of this writing, fluorescence polarization plate readers for 1536 wells are available – but one can expect that number to increase in the near future.

INFLUENCE OF SCATTERING

If a sample contains large molecular assemblies one may have to be concerned with light scattering. Sample turbidity often occurs in membrane systems or in systems containing large macromolecules, such as protein aggregates or large DNA molecules. In these cases one may have to consider the effect of scattering on the measured polarization. Before considering the intrinsic effect of scattering on polarization, however, one must be certain that trivial instrumental factors have been eliminated. By these we mean the case of unwanted light reaching the detector. Unfortunately, optical filters and monochromators are not perfect devices, and hence a small amount of light may pass through these devices, which are intended to block unwanted wavelengths. For non-turbid, *i.e.*, optically clear samples, stray light is not usually a problem since the typical right-angle observation geometry already serves to isolate the emission from the excitation. If a sample scatters appreciably, however, then unwanted or parasitic light may be present. Parasitic light occurs when wavelengths other than the intended range reach the exit slit of the monochromator – such parasitic light peaks are often known as Rayleigh Ghosts and an example is shown in figure **5**, which was obtained many years ago on a 1/4 meter Jarrell-Ash monochromator [31]. In this particular example, the exciting light is 350nm and the two peaks shown in the upper trace (solid circles) in figure **5** are actually 350nm light, which reached the exit slit of the monochromator (we should note that the vertical scale is greatly expanded to detect the signals which are relatively weak). When a cut-on filter, which blocked wavelengths below 400nm, was placed before the emission monochromator, these Rayleigh Ghosts were exorcized (the lower trace – open circles - in figure **5**). Since the advent of holographically ruled diffraction gratings, the problem of parasitic light has been greatly reduced (although polarization artifacts such as Wood's Anomaly are still present in modern monochromators and still confuse the unwary fluorescence practitioner; for a discussion of these issues see [32]). Still, if polarization measurements are required on turbid samples one must be diligent to check for parasitic light since it will be highly polarized and can seriously affect the measurement. In his classic paper on sources of error in polarization measurements [27], Weber described the correct method to check for the presence of parasitic exciting light from the excitation monochromator reaching the emission detector, in the case of a filter-based system. Namely, one simply inserts in the excitation pathway the cut-on filter used on the emission side to isolate the fluorescence. Then, one should observe only the dark signal since no exciting light should be reaching the sample. The presence of a signal on the emission side means either that there is parasitic light coming from the excitation monochromator, or that the filter choice is inherently poor and is not blocking the specified wavelengths. Another way to greatly eliminate parasitic light is to use double

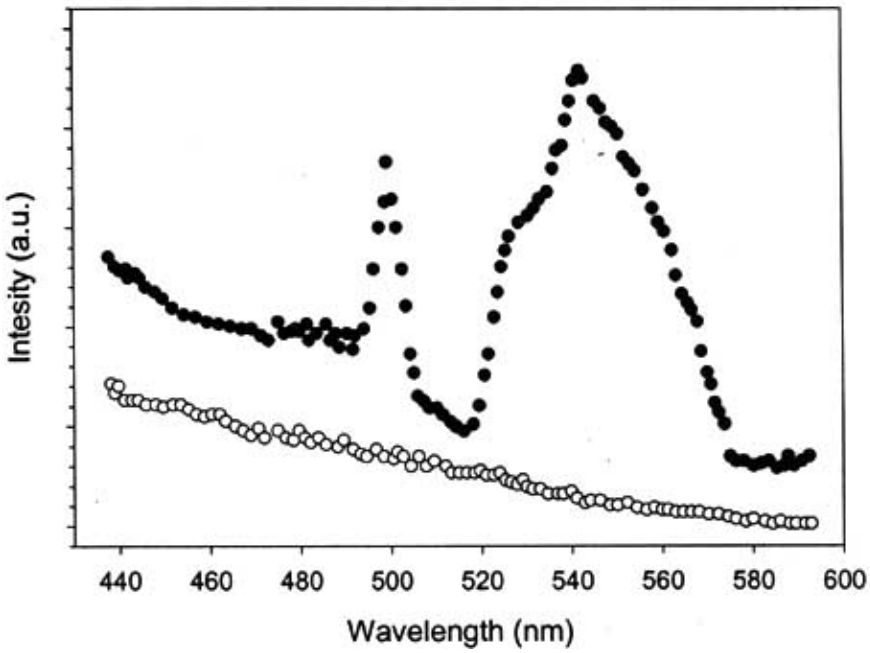

Fig. (5). Demonstration of parasitic light (Rayleigh Ghosts) in a _ meter Jarrell-Ash monochromator (data was obtained on the photon-counting instrument described in [9]). Upper trace (closed circles) - buffer solution, excited at 350nm and scanned from 436-595nm. Lower trace (open circles) - the same scan but with a cut-on filter, which blocked wavelengths below 400nm, placed between the sample and the emission monochromator to prevent the 350nm scatter from entering the monochromator.

monochromator systems – but of course this approach greatly reduces the signal levels. Finally, one can utilize blank subtraction to correct for any spurious light reaching the detector.

If one has eliminated all sources of parasitic light, what then will be the effect of sample turbidity on the observed polarization? In fact, turbidity, which is due to multiple scattering events, will always tend to decrease the polarization. Both excitation and emitted light will essentially be rotated as a consequence of multiple scattering effects, which leads to the diminished polarization. This effect has been studied in detail, both theoretically and empirically, by Teale [33]. The magnitude of this effect can be significant, e.g., for the case of dansyl labeled bovine serum albumin the observed polarization (excited at 366nm) decreased from 0.306 to 0.250 when 0.7% glycogen was added which increased the optical density of the solution from 0.02 to only 0.3 [33]. If turbidity is inherent in the sample, one can reduce the effect of scattering by reducing the light path, i.e., instead of using the normal 1 cm pathlength cuvets one can use cuvets of smaller dimensions (e.g., 3 mm square). We should emphasize that blank subtraction, i.e., subtracting the signal due to the scattering solution without fluorophore, will not correct for the intrinsic depolarization of the emission by scattering processes.

EFFECT OF ENERGY TRANSFER ON POLARIZATION

Depolarization of fluorescence generally occurs either because of Brownian rotations (as discussed earlier) or because of fluorescence resonance energy transfer (FRET)[4]. In this latter case, the excited fluorophore transfers energy, in a radiationless process, to an acceptor - with absorption and emission dipole moments oriented differently from the donor molecule - which in turn emits a photon. Although one usually thinks of FRET occurring between donor and acceptor molecules which differ chemically from one another, the first observation of non-radiative resonance energy transfer was by Gaviola and Pringsham [34] who noted that an increase in the concentration of fluorescein in viscous solvent was accompanied by a progressive depolarization of the emission. The theory of depolarization due to self-transfer (homo-FRET) was further developed by several investigators over the years, including Perrin, Förster and Weber (for a recent review of theory and experimental results see [32]). Homo-FRET has been used to study intrinsic protein fluorescence [35], *in vitro* [36-40] and *in vivo* [41] protein interactions, and peptide and protein associations in membrane systems [42,43]. Interestingly, Weber found that the efficiency of self-transfer drops off dramatically when one excites the fluorophore at the red-edge (i.e., low energy end) of its absorption spectrum [35,44]. This phenomenon, known as the Weber Red Edge Effect, can be useful as an "internal" standard for homo-FRET studies [39,45]. A recent discussion of red-edge effects is given in Bernard Valeur's excellent book [46].

[4] As pointed out to the authors recently by Robert Dale, the term "Fluorescence Resonance Energy Transfer" is somewhat a misnomer since the donor molecule – i.e., the one doing the transferring – does not emit fluorescence if FRET occurs. Dale suggests that the term FRET would more properly stand for Förster Resonance Energy Transfer", to honor Theodore Förster, who, along with Jean and Francis Perrin, formulated the theory of non-radiative energy transfer.

MULTIPHOTON APPLICATIONS

The vast majority of traditional fluorescence determinations are accomplished by the absorption of one photon – typically with an energy near the absorption maximum of the fluorophore. However, as long ago as 1931, the famous physicist Maria Goeppert-Mayer predicted the possibility of non-linear, multi-photon absorption [47]. The 1990 article by Denk, Strickler and Webb [48], however, ushered in the modern use of multi-photon microscopy. If the excitation light density is sufficiently high – which can now be routinely achieved using ultra-short pulsed (femtosecond), high peak power (kilowatt) laser sources such as the Titanium:Sapphire laser (which emits in the near-infrared, over a range of around 700 – 1000nm) – then two photons, each at approximately half the wavelength of the normal (*i.e.*, one photon) absorption of the target chromophore – can be simultaneously absorbed (we say "approximately" since the shape and position of a fluorophore's two-photon cross-section can differ somewhat from that expected from simple consideration of the one-photon absorption spectrum). For an excellent review of the fundamentals, see [49]. This process is illustrated schematically in Figure **6**. The drawing on the left side of figure **6B** depicts a typical light density profile generated by a microscope objective – one notes that exciting light, although maximal at the focal point, persists throughout a larger area, which means that fluorophores both above and below the focal plane are excited. Fluorescence from the focal plane must then be isolated using traditional confocal optics. The drawing on the right side of figure **6B** attempts to depict the fact, in the case of two-photon excitation, that only at the focal point is the light density sufficiently high to generate the two-photon effect so that fluorophore excitation occurs only in a small volume. The advantages of multi-photon excitation are manifold, including [1] a very large separation between the excitation and emission wavelengths (which greatly minimizes problems associated with scattered light), [2] an intrinsic confocal aspect, and [3] reduced overall photobleaching, since light outside of the focal region is at a wavelength not usually absorbed by the sample. Interestingly, the limiting polarization is also higher than the 0.5 level attained in one-photon excitation. In fact, the limiting polarization can reach 0.67 for two-photon excitation and 0.75 for three-photon excitation! The reason for these higher limits is due to the fact that each photon absorbed carries out a photoselection process so that the actual probability of absorption becomes dependent on $\cos^4 \theta$ (in the case of two photons) and $\cos^6 \theta$ (in the case of three photons) where θ is the angle between the transition absorption dipole of the fluorophore and the direction of the electric vector of the exciting light (see, for example, [50]). This principle is illustrated schematically in figure **2B**.

Although many research laboratories now routinely use multiphoton fluorescence to study myriad phenomena, including "optical tweezers", the method has not found significant (if at all) application in diagnostic or high-

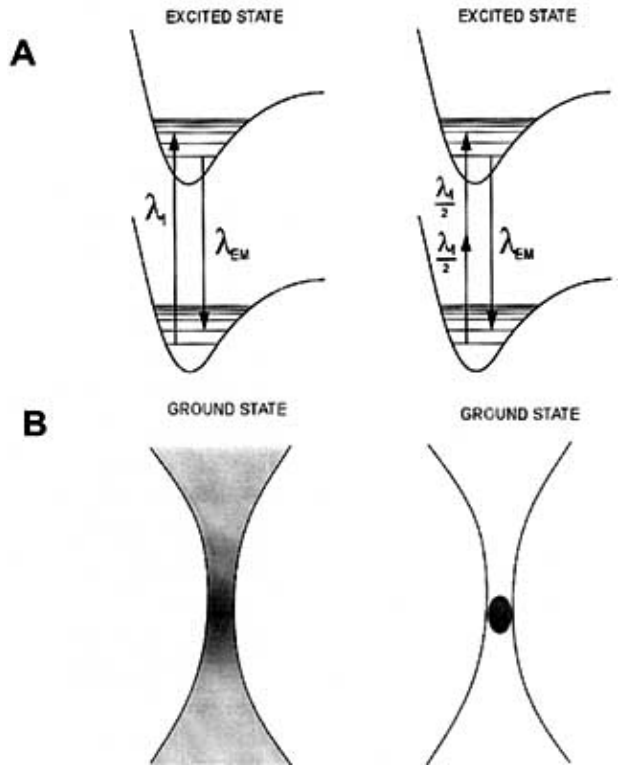

Fig. (6). Depiction of one and two-photon absorption processes.
A. Energy level diagrams depicting (left) one photon absorption and emission and (right) two-photon absorption and emission.
B. Depiction of effective excitation light energy in a volume element illuminated by a microscope objective for (left) one photon and (right) two-photon processes.

throughput procedures (at least at the time these words were written!). Despite the advantages multiphoton methods offer, i.e., higher polarizations, less sample photobleaching and far less scattered light, the higher costs attendant to the method may, at present, be prohibitive. However, as advances in optics and lasers continue to reduce costs, one can predict that it is only a matter of time before multi-photon methods are routinely used in all aspects of biomedical fluorescence.

SUMMARY

This brief overview of fluorescence polarization theory and practice is clearly not exhaustive. But we hope we have given some readers an appreciation for the early development of the method and some of practical considerations. Readers with a sustaining interest should consult some of the more comprehensive sources such as Molecular Fluorescence: Principles and Applications, an excellent new text by Bernard Valeur [46] or Principles of Fluorescence by Lakowicz [50]. Many specialized fluorescence topics are covered in the series Topics in Fluorescence Spectroscopy (Volumes 1-6), and several volumes of Methods in Enzymology (e.g., volumes 246, 278 and the upcoming volumes 360 and 361) have dealt with issues in fluorescence spectroscopy. Proceedings from the MAFS7 [51] and MAFS8 [52] conferences present fluorescence work on many different topics in the biological and chemical fields, and the Molecular Probes Handbook and web site (http://www.probes.com/) are also rich sources of useful information. Finally, any reader with a question on some topic related to fluorescence is welcome to e-mail DMJ at djameson@hawaii.edu.

ACKNOWLEDGEMENT

We wish to thank Oliver Holub for the English translation of the original Weigert manuscript, and Yuling Yan and Gerard Marriott for valuable discussions on the numerical aperture effect. DMJ acknowledges support from the American Heart Association (0151578Z).

REFERENCES

[1] Weigert, F. *Verh. d.D. Phys. Ges.* **1920**, *1*, 100-102.
[2] Vavilov, S. J.; Levschin, W. L. *Z. Physik.* **1923**, *16*, 135-154.
[3] Perrin, F. *Comptes Rendues* **1925**, *180*, 581-583.
[4] Perrin, F. *Comptes Rendues* **1925**, *181*, 514-516.
[5] Perrin, F. *J. Physique* **1926**, *7*, 390-401.
[6] Berberan-Santos, M. N. In *New Trends in Fluorescence Spectroscopy*; Valeur, B., Brochon, J.-C. Eds.; Springer: Heidelberg, **2001**, pp 7-33.
[7] Weber, G. In *Fluorescent Biomolecules*; Jameson, D. M.; Reinhart, G. D. Eds.; Plenum Press: New York, **1989**, pp 343-349.
[8] Jameson, D. M. In *New Trends in Fluorescence Spectroscopy*; Valeur, B.; Brochon, J.-C. Eds.; Springer: Heidelberg, **2001**, pp 35-58.
[9] Jameson, D. M.; Weber, G.; Spencer, R. D.; Mitchell, G. *Review of Scientific Instruments* **1978**, *49*, 510-514.
[10] Azumi, T.; McGlynn, S. P. *J. Phys. Chem.* **1962**, *37*, 2413-2420.
[11] Jameson, D. M.; Sawyer, W. H. *Methods Enzymol.* **1995**, *246*, 283-300.
[12] Laurence, D. J. R. *Biochem. J.* **1952**, *51*, 168-177.
[13] Dandliker, W. B.; Feijen, G. A. *Biochem. Biophys. Res. Comm.* **1961**, *5*, 299-304.
[14] Dandliker, W. B.; De Saussure, V. A. *Immunochemistry* **1970**, *7*, 799-828.
[15] Spencer, R. D.; Toledo, F. B.; Williams, B. T.; Yoss, N. L. *Clin. Chem.* **1973**, *8*, 838-844.
[16] Jolley, M. E.; Stroupe, S. D.; Schwenzer, K. S.; Wang, C. J.; Lu-Steffes, M.; Hill, H. D.; Popelka, S. R.; Holen, J. T.; Kelso, D. M. *Clin. Chem.* **1981**, *27*, 1575-1579.
[17] Jolley, M. E.; Stroupe, S. D.; Wang, C. H.; Panas, H. N.; Keegan, C. L.; Schmidt, R. L.; Schwenzer, K. S. *Clin. Chem.* **1981**, *27*, 1190-1197.
[18] Jolley, M. E. *J. Anal. Toxicol.* **1981**, *5*, 236-240.
[19] Nasir, M. S.; Jolley, M. E. *Comb. Chem. High Throughput Screen.* **1999**, *2*, 177-190.
[20] Weber, G. *Biochem. J.* **1952**, *51*, 145-155.
[21] Jablonski, A. *Bull. Acad. Polon. Sci. Serie des sci. math. astr. et phys.* **1960**, *6*, 259-264.
[22] Mocz, G.; Helms, M. K.; Jameson, D. M.; Gibbons, I. R. *Biochemistry* **1998**, *37*, 9862-9869.
[23] Perrin, F. *Photochimie Ann. Rev. (Paris)* **1929**, *12*, 169-275.
[24] Weber, G. In *Fluorescence and Phosphorescence*; Hercules, D. Ed.; Wiley: New York, **1966**, pp 217-240.
[25] Valeur, B. *Molecular Fluorescence: Principles and Applications*; Wiley-VCH: Weinheim, Germany, **2002**.
[26] Hall, R. D.; Valeur, B.; Weber, G. *Chem. Phys. Lett.* **1985**, *116*, 202-205.
[27] Weber, G. *J. Opt. Soc. Amer.* **1956**, *46*, 962-970.
[28] Axelrod, D. *Biophys. J.* **1979**, *26*, 557-573,.
[29] Jovin, T. M. In *Flow Cytometry and Sorting*; Melamed, Mullamey'Mendelson, Eds.; John Wiley & Sons: New York, **1979**.
[30] Yan, Y.; Marriott, G. *Methods Enzymol.* **2003**, *360*, 561-580.
[31] Jameson, D. M. In *Biochemistry*; UIUC, **1978**.
[32] Jameson, D. M.; Croney, J. C.; Moens, P. D. J. *Methods Enzymol.* **2003**, *360*, 1-43.
[33] Teale, F. W. J. *Photochem. Photobiol.* **1969**, *10*, 363-374.
[34] Gaviola, E.; Pringsheim, P. *Z. Physik* **1924**, *24*, 24-36.
[35] Weber, G. *Biochemical Journal* **1960**, *75*, 345-352.
[36] Ruan, K.; Weber, G. *Biochemistry* **1993**, *32*, 6295-6301.
[37] Erijman, L.; Weber, G. *Biochemistry* **1991**, *30*, 1595-1599.
[38] Erijman, L.; Weber, G. *Photochem. Photobiol.* **1993**, *57*, 411-415.
[39] Hamman, B. D.; Oleinikov, A. V.; Jokhadze, G. G.; Traut, R. R.; Jameson, D. M. *Biochemistry* **1996**, *35*, 16680-16686.
[40] Scarlata, S.; Ehrlich, L. S.; Carter, C. A. *J. Mol. Biol.* **1998**, *277*, 161-169.
[41] Gautier, I.; Tramier, M.; Durieux, C.; Coppey, J.; Pansu, R. B.; Nicolas, J. C.; Kemnitz, K.; Coppey-Moisan, M. *Biophys. J.* **2001**, *80*, 3000-3008.
[42] MacPhee, C. E.; Howlett, G. J.; Sawyer, W. H.; Clayton, A. H. *Biochemistry* **1999**, *38*, 10878-10884.
[43] Blackman, S. M.; Piston, D. W.; Beth, A. H. *Biophys. J.* **1998**, *75*, 1117-1130.
[44] Weber, G.; Shinitsky, M. *Proc. Natl. Acad Sci. USA* **1970**, *65*, 823-830.
[45] Helms, M. K.; Hazlett, T. L.; Mizuguchi, H.; Hasemann, C. A.; Uyeda, K.; Jameson, D. M. *Biochemistry* **1998**, *37*, 14057-14064.
[46] Valeur, B. Molecular Fluorescence: Principles and Applications; Wiley-VCH: Weinheim, **2002**.
[47] Goeppert-Mayer, M. *Ann. Physik* **1931**, *9*, 273-294.

[48] Denk, W.; Strickler, J. H.; Webb, W. W. *Science* **1990**, *248*, 73-76.
[49] Nakamura, O. *Microscopy Res. Tech.* **1999**, *47*, 165-171.
[50] Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Second ed.; Kluwer Academic: New York, **1999**.
[51] Valeur, B.; Brochon, J.-C. Eds. *New Trends in Fluorescence Spectroscopy*; Springer: Heidelberg, **2001**.
[52] Visser, T.; Kraayenhof, R. Eds. *Methods and Apllications in Fluorescence Spectroscopy*; Springer: Berlin, In Press.