Simultaneous Measurement of Circular Dichroism and Fluorescence Polarization Anisotropy

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Keywords: circular dichroism, fluorescence polarization anisotropy, ultraviolet absorption spectra, photoelastic modulator, stopped flow kinetics

ABSTRACT

Circular dichroism and fluorescence polarization anisotropy are important tools for characterizing biomolecular systems. Both are used extensively in kinetic experiments involving stopped- or continuous flow systems as well as titrations and steady-state spectroscopy. This paper presents the theory for determining circular dichroism and fluorescence polarization anisotropy simultaneously, thus insuring the two parameters are recorded under exactly the same conditions and at exactly the same time in kinetic experiments. The approach to measuring circular dichroism is that used in almost all conventional dichrographs. Two arrangements for measuring fluorescence polarization anisotropy are described. One uses a single fluorescence detector and signal processing with a lock-in amplifier that is similar to the measurement of circular dichroism. The second approach uses classic "T" format detection optics, and thus can be used with conventional photon-counting detection electronics. Simple extensions permit the simultaneous measurement of the absorption and excitation intensity corrected fluorescence intensity.

INTRODUCTION

Visible and ultraviolet light are important tools in biomedical research. The absorption of a sample is the most fundamental physical effect, but numerous related phenomena extend the range of information obtainable from a sample. These include:

- elastic and inelastic scattering of incident radiation, which are alternates to the absorption of an incident photon,
- reemission of absorbed photon energy, *i. e.*, fluorescence, phosphorescence, or more generally luminescence,
- the polarization of absorbed, scattered or emitted photons.

Fluorescence and other forms of luminescence are powerful and widely used tools because they offer several observable parameters: the spectrum of the emitted light, the spectrum that stimulates the emission, and the temporal course of the emission. Polarization measurements, either linear or circular, provide additional information on all three basic photonic interactions: scattering, absorption and luminescence. While both linear and circular polarization measurements have been applied for each class of photonic interactions, measurements of linear polarization effects are most prevalent for scattering and luminescence, while circular polarization tends to be more important in absorption spectroscopy. One reason is that an absorbing species will differentiate between the linear polarization states of an incident photon only if there is some spatial alignment of the sample, but randomly oriented absorbing species can absorb left- and right-circularly polarized photons differently, based on either inherent or induced asymmetries. In contrast, scattered or emitted light can be linearly polarized as a result of the linear polarization state of the photons incident on randomly oriented samples.

The plethora of potentially useful experimental measurements creates a conundrum for scientists in the selection of spectroscopic instruments. How do we acquire a broad range of experimental capabilities consistent with practical constraints such as budgets and space limitations. A particularly useful combination is to combine circular dichroism and fluorescence capabilities because both these experiments require an intense, broad spectrum light source like a xenon arc[1] or a synchrotron storage ring.[2] In addition, circular dichroism instruments almost always employ a phase-sensitive detector (lock-in amplifier) for the extraction of small signals and fluorometers can benefit from this form of signal

processing. Advances in computer technology made it possible to record circular dichroism and absorption[3] or fluorescence at the same time.[4,5] In the case of a synchrotron source, which generates pulses of light at a high frequency, it is also possible to measure the time course of fluorescence using single photon counting in the same instrument.[6]

In this paper, I present a theory for the simultaneous measurements of circular dichroism and fluorescence polarization anisotropy, the two most widely used forms of polarization measurements in biophysical photonics. The challenge in developing this capability is that one involves circularly and the other involves linearly polarized light. Using previously reported approaches, these capabilities can be extended to also include quantum corrected fluorescence intensity and absorption. I expect that the primary impact of this capability will be in determining the response of a test system as a function of time as, *e. g.*, in a stopped flow experiment, or as part of a titration, rather than in the recording of spectra. However, the basic technology described herein can also be used in an economical instrument capable of measuring either circular dichroism or fluorescence polarization anisotropy while sharing most major optical and electronic components.

THEORY

Fluorescence Polarization

A diagram of one configuration of components presently used to measure fluorescence polarization is shown in Figure 1. The vertically polarized incident beam of photons of wavelength λ excites fluorescence from the sample, which is filtered to remove scattered incident light (filters not shown) and also passes through polarizers before reaching detectors that record the two orthogonal linear polarization components of the fluorescence. These beams of fluorescence are vertically and horizontally polarized.

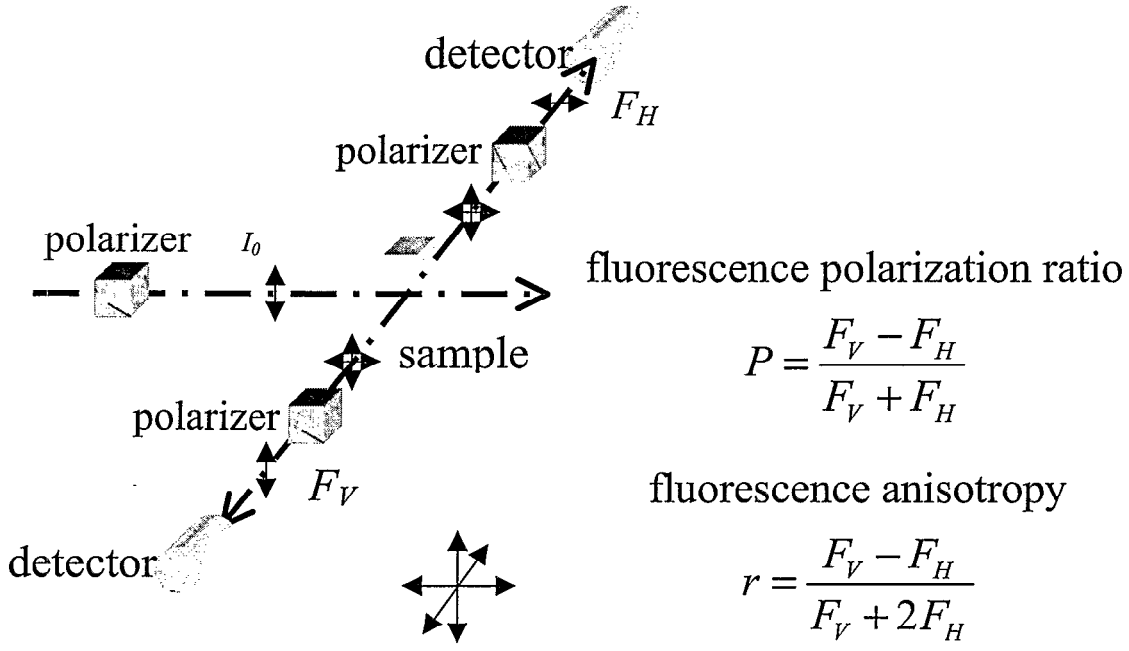

Figure 1 Schematic diagram for the measurement of fluorescence polarization anisotropy using two detection channels (T format). The plane defined by the incident photon beam and the orthogonal direction between the sample and the fluorescence detectors is defined as the horizontal plane, so the incident beam is characterized as vertically polarized.

The degree of polarization of the fluorescence from a sample can be characterized either by the polarization ratio, P, or the anisotropy ratio, r, which is preferable for reasons explained in standard references on fluorescence.[7] Both P and r

are self-normalizing. That is, the terms in both numerator and denominator are proportional to the intensity of the incident light, so the ratio is independent of the incident intensity (although the signal-to-noise ratio is strongly dependent on incident intensity). Rotating the polarizer in the incident beam (the excitation polarizer) by $\pi/2$ causes both emission channels to record a signal proportional to F_H, an effect commonly used in calibrating the responses of emission channels in both T and L format fluorometers.[7] Rotation of the excitation polarizer cannot, however, be used in the measurement of P and r along the lines described below because the monochromatic photon beam from most experimental sources, particularly those employing a monochromator, are partially polarized. In this situation, changing the incident polarization also changes the intensity, hence destroying the self-normalizing property of the measurement.

For the experimental arrangement shown in Figure 1, P and r are measured by the ratios of fluorescence intensity indicated in the figure. A definition that will be more useful in this work involves defining these parameters in terms of the quantum yields for fluorescence parallel and perpendicular to the direction of polarization of the exciting light, as shown for r in Equation 1, irrespective of the actual direction of that vector in space.

$$r = \frac{\phi_{\parallel} - \phi_{\perp}}{\phi_{\parallel} + 2\phi_{\perp}} \qquad \text{Equation 1}$$

Dupont and his colleagues[8] pointed out that if the emission polarizer is removed from one of the emission channels shown in Figure 1 then the signal received when the excitation polarization is vertical is $F_V = F_{\parallel} +, F_{\perp}$, while when the excitation polarization is horizontal, the intensity recorded in the same channel would by $F_H = 2\,F_{\perp}$, where $F_{\parallel}$ and $F_{\perp}$ are intensities emitted parallel and perpendicular to the direction of polarization of the exciting light, and the subscripts "*V*" and "*H*" refer to the polarization of the excitation beam. Were it not for the change in intensity of the excitation beam that occurs, rotating the excitation polarizer would provide enough information to determine P and r.

Circular Dichroism

A schematic diagram of components used for measuring circular dichroism is shown in Figure 2. The incident photon beam contains a polarizer, as does the fluorometer shown in Figure 1. However, a photoelastic modulator is placed between the polarizer and the sample and a detector monitors the beam transmitted through the sample. The photoelastic modulator changes the polarization of the photons reaching the sample without changing the total photon flux. Because the detector (ideally) responds only to the total photon flux and not to the polarization of these photons, the signal reaching the detector is constant unless the sample absorbs (or scatters) the transmitted beam differentially as a function of polarization.

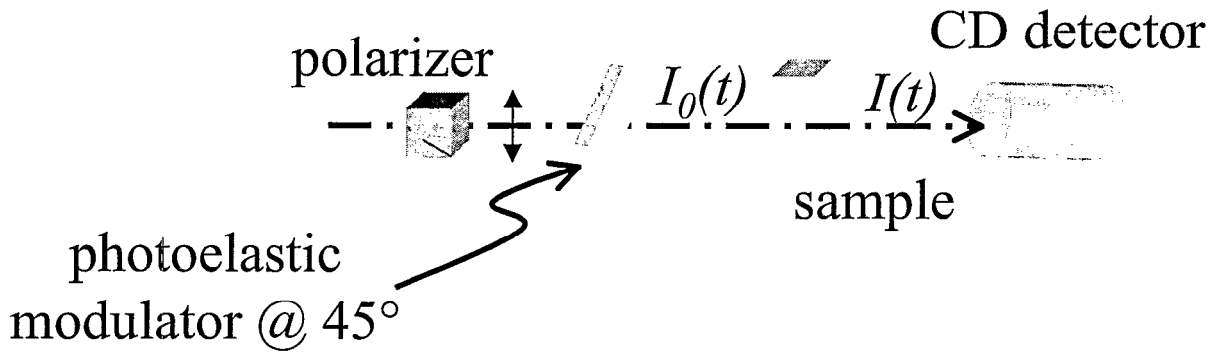

Figure 2 Schematic diagram of an instrument for the measurement of circular dichroism.

Assuming that a vertically polarized beam is incident on the photoelastic modulator and the strain axis of the modulator is oriented at $\pi/4$ radians off vertical, the photoelastic modulator will produce a phase shift δ between the component of the incident light parallel to its stress axis compared to the component perpendicular to that axis.[9,10] Photoelastic modulators are resonant devices so the differential phase retardation changes periodically at the frequency of the modulator (typically 50 kHz) according to Equation 2, where d is the thickness of the modulator element, S is the strain

$$\delta(t)=\frac{2\pi d}{\lambda}C\,S\sin(\omega t)=\delta_0\sin(\omega t) \qquad \text{Equation 2}$$

produced by the modulator drive system, and C is the stress optical constant to the modulator drive element at wavelength λ. The product of the factors multiplying the sine term is designated δ_0, the value of which can be controlled by changing the value of S by means of the modulator drive circuit. δ_0 is the maximum amplitude of the phase shift induced by the photoelastic modulator. The beam emerging from the photoelastic modulator can be described equally well as the sum of two linearly polarized components or two circularly polarized components as shown in Equation 3 and Equation 4, where the subscripts L and R designate left and right and, as above, V and H denote vertical and horizontal. The plus and minus signs apply to the first and second subscripts, respectively.

$$\text{Linear: } I_0(t)=I_V(t)+I_H(t), \quad \text{where } I_{V/H}(t)=\frac{I_0}{2}\left(1\pm\cos\left[\delta(t)\right]\right) \qquad \text{Equation 3}$$

$$\text{Circular: } I_0(t)=I_L(t)+I_R(t), \quad \text{where } I_{L/R}(t)=\frac{I_0}{2}\left(1\pm\sin\left[\delta(t)\right]\right) \qquad \text{Equation 4}$$

The trigonometric functions in these two equations are expanded using the expression for $\delta(t)$ from Equation 2 in terms of Bessel functions of the first kind as shown in Equation 5 and Equation 6. Thus the description of the photon beam incident of the sample in terms of linearly polarized light depends only on the Bessel functions of even orders while the description based on circularly polarized components depends only on the odd orders. The values of the three lowest order Bessel functions are shown in Figure 3.

$$\cos\left(\delta_0\sin(\omega t)\right)=J_0(\delta_0)+2J_2(\delta_0)\cos(2\omega t)+2J_4(\delta_0)\cos(4\omega t)+\ldots \qquad \text{Equation 5}$$

$$\sin\left(\delta_0\sin(\omega t)\right)=2J_1(\delta_0)\sin(\omega t)+2J_3(\delta_0)\sin(3\omega t)+\ldots \qquad \text{Equation 6}$$

The approach is to determine how the sample will affect the absorption and/or emission of the separate polarization components and then sum their effects on the total photon flux reaching a detector, $I(t)$. In the case of circular dichroism, different absorption coefficients, A_L and A_R, are applied to the two corresponding intensity components as shown in Equation 7.

$$I(t)=I_L(t)10^{-A_L}+I_R(t)10^{-A_R} \qquad \text{Equation 7}$$

Assuming that the sample does not exhibit linear dichroism, *i. e.*, $A_V = A_H$, and that the circular dichroism is small compared to the average absorption, *i. e.*, $A_L - A_R \ll (A_L+A_R)/2$, we expand the exponential terms in Equation 7 in a Taylor series, discarding all terms higher than first order. We also expand the two intensity terms according to Equation 4 and Equation 6, discarding all terms in frequencies greater than ω, obtaining the result shown in Equation 8.

$$I(t)=I_0(\lambda)10^{-\left(\frac{A_L+A_R}{2}\right)}\left[1-\ln(10)J_1(\delta_0)(A_L-A_R)\sin(\omega t)\ldots\right] \qquad \text{Equation 8}$$

The signal reaching the detector can be described as the sum of an unmodulated or "dc" component, $\overline{I}$, a component modulated at the fundamental frequency of the photoelastic modulator, ΔI_ω, plus terms modulated at the odd harmonics of the modulator frequency according to Equation 9.

$$I(t)=\overline{I}+\Delta I_\omega\sin(\omega t)+\ldots \qquad \text{Equation 9}$$

The value of $\overline{I}$ is obtained in an experiment by passing the electronic signal from the detector through a low pass filter, while , ΔI_ω is obtained from the output of a phase sensitive detector (lock-in amplifier) tuned to the frequency and phase of the photoelastic modulator. Comparing corresponding terms in Equation 8 and Equation 9 gives the expression for circular dichroism shown in Equation 10. The expression in parenthesis represents a calibration factor for the circular

$$A_L-A_R=\left(\frac{-1}{\ln(10)J_1(\delta_0)}\right)\frac{\Delta I_\omega}{\overline{I}} \qquad \text{Equation 10}$$

dichroism experiment. Contrary to what might be expected, the modulator need not be operated to produce quarter wave retardation ($\delta_0 = \pi/2$) for the measurement of circular dichroism. Indeed, the only formal restriction is that the phase must be chosen such that $J_1(\delta_0) \neq 0$. For a given value of the circular dichroism of a sample, the maximum value of ΔI_ω and hence the minimum value of the calibration constant will result for the value of δ_0 that corresponds to the maximum value of J_1, which is approximately 0.587 π (106°). In a properly calibrated circular dichroism spectrometer, other values of δ_0 will result in a smaller experimental value of ΔI_ω being multiplied by a correspondingly larger calibration constant. While not affecting the accuracy of a circular dichroism measurement, choosing the phase amplitude at the maximum of the J_1 function optimizes the signal-to-noise ratio, and hence the precision of the measurement.

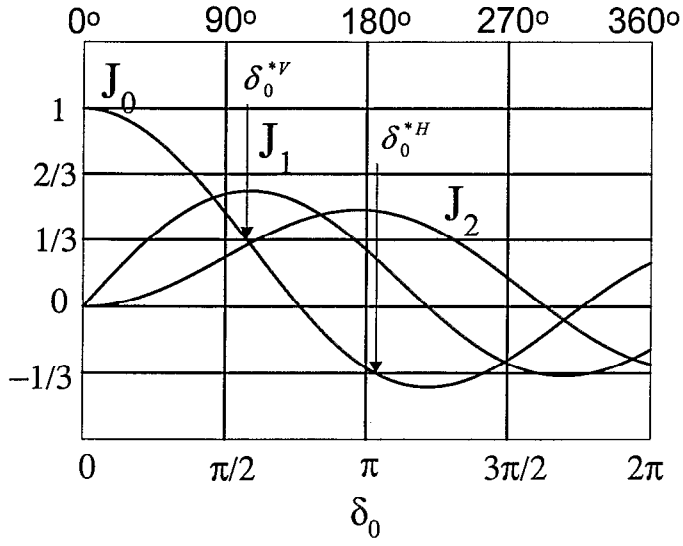

Figure 3 The three lowest order Bessel functions of the first kind as a function of the maximum phase amplitude, δ_0. The values of δ_0 that cause J_0 to equal 1/3 and −1/3 are designated "**magic phase**" amplitudes. They are critical to measurements of fluorescence polarization anisotropy, as described in the text, and represented by the symbols δ_0^{*V} and δ_0^{*H} respectively.

Simultaneous Circular Dichroism and Fluorescence Polarization

A schematic diagram of an instrument to measure both circular dichroism and fluorescence polarization at the same time is shown in Figure 4. The arrangement of optical components along the transmission axis is identical to that for a circular dichroism experiment as shown in Figure 2. Note that only one fluorescence detector is required and no polarizer is present between the sample and this detector.

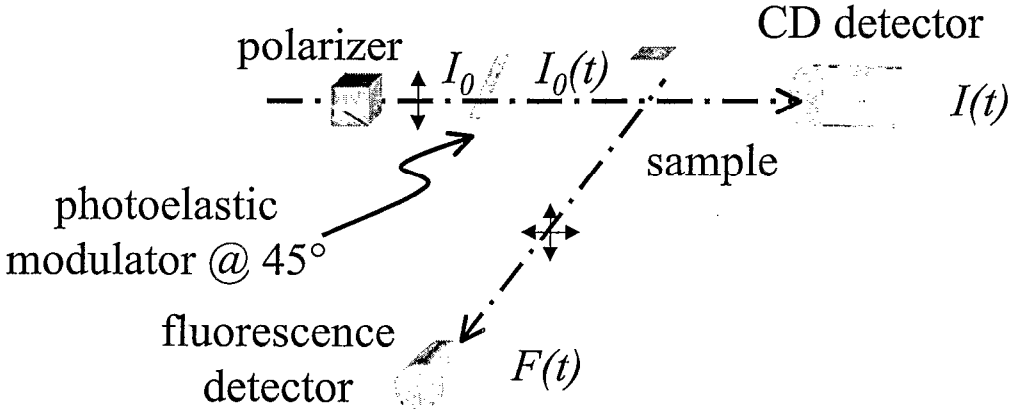

Figure 4 Schematic diagram of an instrument for the simultaneous measurement of circular dichroism and fluorescence polarization. A polarization-neutral filter to prevent scattered incident light from reaching the fluorescence detector is required, although not shown.

As with circular dichroism, we presume that the sample absorbs the two linearly polarized components of the incident beam to the same degree, *i. e.*, there is no linear dichroism so $A_H = A_V = A$. Thus, any linear polarization of the fluorescence must result from differences in quantum yield for emission parallel and perpendicular to the polarization of the incident beam, usually due to incomplete depolarization of the fluorescing species during the lifetime of the excited state – typically a few ns. In the absence of an emission polarizer, the detector records light emitted parallel and perpendicular to the direction of polarization of the incident beam for the vertically polarized component of the exciting beam. But when the incident beam is horizontally polarized, both the horizontally and vertically polarized components of fluorescence reaching the detector are emitted perpendicular to the polarization of the exciting beam. Thus, the time varying fluorescence intensity reaching the detector is described by Equation 11, which presumes that the detector collects fluorescence from all portions of the sample with equal efficiency.

$$F(t) = \left[\left(\phi_{\parallel} + \phi_{\perp} \right) I_V(t) + 2\phi_{\perp} I_H(t) \right] \left(1 - 10^{-A} \right) \qquad \text{Equation 11}$$

Substituting the expressions for the vertically and horizontally polarized intensities shown in Equation 3 and the expansion for $\cos(\delta_0 \sin(\omega t))$ from Equation 5 gives the result shown in Equation 12.

$$F(t) = \left(1 - 10^{-A}\right) \frac{I_0}{2} \left\{ \phi_{\parallel} \left[1 + J_0(\delta_0) \right] + \phi_{\perp} \left[3 - J_0(\delta_0) \right] + 2 \left[\phi_{\parallel} - \phi_{\perp} \right] J_2(\delta_0) \cos(2\omega t) + \ldots \right\} \qquad \text{Equation 12}$$

The signal generated by the fluorescence detector can be described as the sum of a time invariant signal, $\overline{F}$, a signal modulated at twice the frequency of the photoelastic modulator, $\Delta F_{2\omega}$, plus a series of terms modulated at the higher even harmonics of the photoelastic modulator frequency, as shown in Equation 13.

$$F(t) = \overline{F} + \Delta F_{2\omega} \cos\left(2\omega t\right) + \ldots \qquad \text{Equation 13}$$

$\overline{F}$ can be obtained by passing the voltage generated by $F(t)$ through a low pass filter or by averaging it over many cycles of the photoelastic modulator, while $\Delta F_{2\omega}$ is obtained from a lock-in amplifier having the $F(t)$ generated voltage as input and tuned to twice the frequency of the photoelastic modulator. Note that in both this situation and in the case of circular dichroism considered above, we do not need to assume that the higher harmonics are of insignificant amplitude. Rather, they are not detected by these procedures. Comparing Equation 12 and Equation 13 leads to Equation 14.

$$\frac{\Delta F_{2\omega}}{\overline{F}} = \frac{2\left[\phi_{\parallel} - \phi_{\perp} \right] J_2(\delta_0)}{\phi_{\parallel} \left[1 + J_0(\delta_0) \right] + \phi_{\perp} \left[3 - J_0(\delta_0) \right]} \qquad \text{Equation 14}$$

In a measurement of fluorescence polarization anisotropy, the intensity of fluorescence polarized perpendicular to the polarization of the exciting light must be weighted twice as heavily as the parallel polarized component. This can be accomplished by choosing δ_0 such that $J_0 = 1/3$. The (smallest) phase amplitude that fulfills this condition is designate by the symbol δ_0^{*V}. I call this the **magic phase** amplitude because it causes fluorescence polarized perpendicular to the polarization of the exciting light to be weighted twice as heavily as parallel polarized fluorescence, in analogy with a **magic angle** emission polarizer, which achieve the same weighting.[7] Actually, δ_0^{*V} is the magic phase for vertically polarized exciting light incident on the photoelastic modulator. A different phase amplitude satisfies the magic phase condition if the light incident on the photoelastic modulator is horizontally, rather than vertically polarized (*vide infra*). The value of δ_0^{*V} is approximately 0.577π (104°), which, as shown in Figure 3, is quite close to the phase that produces the maximum value of J_1 and hence the maximum value of ΔI_ω in a CD experiment. Rearranging Equation 14 results in

the expression for fluorescence polarization anisotropy given in Equation 15, where the value of $J_2(\delta_0^{*V})$ is approximately 0.309.

$$r = \frac{\phi_{\parallel} - \phi_{\perp}}{\phi_{\parallel} + 2\phi_{\perp}} = \frac{2\Delta F_{2\omega}}{3J_2(\delta_0^{*V})\bar{F}} \qquad \text{Equation 15}$$

Thus, by adjusting the control circuit to provide a phase retardation amplitude of δ_0^{*V} for the particular excitation wavelength, we can measure circular dichroism by recording the time average and photoelastic modulator fundamental signals ($\bar{I}$ and ΔI_{ω} respectively) using Equation 10, with $\delta_0 = \delta_0^{*V}$ and at the same time measure fluorescence polarization anisotropy, *r*, by monitoring the time average and photoelastic modulator first harmonic signals from the fluorescence detector ($\bar{F}$ and $\Delta F_{2\omega}$ respectively) according to Equation 15.

Simultaneous measurement of circular dichroism and fluorescence polarization anisotropy is also possible if the excitation polarizer is set to pass horizontally polarized light. The major change in analysis is to reverse the meaning of the signs in Equation 3 so that the plus applies to the horizontally polarized beam. The expression analogous to Equation 11 is shown in Equation 16. The criterion that the perpendicularly polarized component is weighted twice as heavily as the parallel component is achieved if $J_0(\delta_0^{*H})$ equals, –1/3 thus defining the magic phase for horizontally polarized excitation, which is approximately 1.035 π (186°), and thus close to the phase amplitude that maximizes J_2. Extraction of the circular dichroism and fluorescence polarization anisotropy proceeds as above, except for changes in the sign of the modulated signals, which can be handled in an experiment by shifting the phase setting of a lock-in amplifier by 180°.

$$F(t) = \left(1-10^{-A}\right)\frac{I_0}{2}\left\{\phi_{\parallel}\left[1-J_0(\delta_0)\right] + \phi_{\perp}\left[3+J_0(\delta_0)\right] - 2\left[\phi_{\parallel}-\phi_{\perp}\right]J_2(\delta_0)\cos(2\omega t)\right\} \qquad \text{Equation 16}$$

Simultaneous Measurement of circular dichroism and fluorescence polarization anisotropy using single-photon counting fluorescence detection.

Single photon counting is an alternate approach to high-sensitivity detection of weak fluorescence signals. Simultaneous detection of circular dichroism and fluorescence polarization anisotropy is compatible with the use of single-photon counting for the fluorescence channel. The configuration for such an experiment involves the circular dichroism experiment just as shown in Figure 2, but with two polarizers and fluorescence detectors as shown in Figure 1. The excitation polarizer is set to produce vertically polarized light. The two fluorescence signals, $F_V(t)$ and $F_H(t)$, will receive fluorescence induced by both the vertically and horizontally polarized components of the exciting beam, as shown in Equation 17 and Equation 18, where $I_V(t)$ and $I_H(t)$ are as defined in Equation 3. Their sum is just I_0, so $F_H(t)$ should not be modulated by the photoelastic modulator, and thus will be written as $\bar{F}_H$.

$$F_V(t) = \left(1-10^{-A}\right)\left[\phi_{\parallel}I_V(t) + \phi_{\perp}I_H(t)\right] \qquad \text{Equation 17}$$

$$F_H(t) = \left(1-10^{-A}\right)\left[\phi_{\perp}I_V(t) + \phi_{\perp}I_H(t)\right] = \left(1-10^{-A}\right)\phi_{\perp}I_0 = \bar{F}_H \qquad \text{Equation 18}$$

We will assume that the fluorescence signals will be accumulated for a period much longer that the period of the photoelastic modulator, (≤ 200 μs is adequite) so the cos(2ωt) and all higher harmonic terms in the expansions for $I_V(t)$ and $I_H(t)$ will average to zero, and the signal reaching the detector after passing through the vertical emission polarizer can be written as shown in Equation 19. Solving for the quantum yields and substituting gives the revised expression for the fluorescence polarization anisotropy appropriate for this experimental configuration shown in Equation 20.

$$\bar{F}_V = \left(1-10^{-A}\right)\frac{I_0}{2}\left[\phi_{\parallel}(1+J_0(\delta_0) + \phi_{\perp}(1-J_0(\delta_0)\right] \qquad \text{Equation 19}$$

$$r = \frac{\phi_{\parallel} - \phi_{\perp}}{\phi_{\parallel} + 2\phi_{\perp}} = \frac{\bar{F}_V - \bar{F}_H}{\bar{F}_V + \frac{\bar{F}_H}{2}(1 + 3J_0(\delta_0))} \qquad \text{Equation 20}$$

Note that when the photoelastic modulator is not operating, $\delta_0 = 0$ and $J_0 = 1$, so r reverts to the classical expression for fluorescence polarization anisotropy shown in Figure 1. In principle, any non-zero value of δ_0 can be used to measure fluorescence polarization anisotropy, but two seem particularly convenient. Setting the phase amplitude so that $J_0 = 1/3$ optimizes the circular dichroism experiment and results for the expression for fluorescence polarization anisotropy shown in Equation 21, while $J_0 = -1/3$ results in Equation 22.

$$r = \frac{\bar{F}_V - \bar{F}_H}{\bar{F}_V + \bar{F}_H}, \; J_0 = \frac{1}{3} \qquad \text{Equation 21}$$

$$r = \frac{\bar{F}_V - \bar{F}_H}{\bar{F}_V}, \; J_0 = -\frac{1}{3} \qquad \text{Equation 22}$$

DISCUSSION

Which Excitation Polarization?

Circular dichroism and fluorescence polarization anisotropy can both be measured accurately with either horizontal or vertical polarization of the incident beam when the latter is detected with a lock-in amplifier. Vertically polarized excitation requires that the maximum phase amplitude be set at the magic value of 0.577π (104°) which is near the phase amplitude that generates the largest circular dichroism signal, hence optimizing the signal-to-noise ratio for this measurement. Horizontal incident polarization, in contrast, requires the magic phase amplitude of $1.035\ \pi$ (186°), which is close to the value that maximizes the fluorescence polarization anisotropy signal, and hence the signal-to-noise ratio for this measurement. This flexibility in the choice of incident polarization is useful in situations in which the incident beam is partially polarized, *e.g.*, when using a synchrotron radiation light source. It must be stressed however, that in both cases, the calibration constants will compensate for the differences in the amplitudes of the modulated signals, so both configurations give accurate measurements. The choice of incident polarization direction only affects the signal-to-noise ratios and hence the precision obtained, and that usually only to a modest degree.

Simultaneous Determination of Absorption and Excitation Corrected Fluorescence.

Both circular dichroism and fluorescence polarization anisotropy are "ratio" measurements and hence self-normalizing. However, both components of the ratio are contained in the intensity of light that has passed through or been emitted from the sample. Thus, neither of these important experimental observables requires we record the intensity of the incident beam. Recording the incident intensity, however, makes it possible to determine two additional important parameters at the same time that circular dichroism and fluorescence polarization anisotropy are measured. These are the absorption* of the sample and the excitation-intensity-corrected fluorescence. The latter is particular useful when excitation spectra are measured, as this correction greatly facilitates comparisons between fluorescence and absorption. Measurement of the absorption of the sample in comparison with a "blank" is based on the "pseudo absorption" technique, which has been described in detail elsewhere.[3,11] The information required for pseudo absorption is less demanding than that required for correcting the fluorescence spectrum, because in can be based on a detector that has a spectral response different than that of the detector used to monitor the circular dichroism. The incident intensity monitor required for the correction of fluorescence, in contrast, should give a signal directly proportional to the incident photon

* Implementations of pseudo-absorption can be achieved without monitoring the intensity of the incident beam if one can reasonably expect the intensity of the light source to remain constant over the period required to measure both the sample and the "blank", and implementations of pseudo-absorption in commercial circular dichroism spectrometers are based on this assumption. However, such an assumption is not justified in most circular dichroism spectrometers that used synchrotron radiation sources, for which it was invented. In any case, having an independent measure of the incident intensity increases the robustness of the method.

flux or a signal that can be converted to photon flux at each incident wavelength. A "quantum screen" is frequently used for this purpose,[7] although the use of this class of detector may limit the spectral region that can be covered. Any detector suitable for obtaining the signal required to correct fluorescence should be adequate for determining pseudo absorption, and many commercial fluorometers include such capabilities.

A Practical Instrument

A diagram of an instrument capable of recording circular dichroism, fluorescence polarization anisotropy, intensity corrected fluorescence and the absorption of a sample is shown in Figure 5. The diagram presumes that it is possible to measure the unmodulated (or "dc") signals from both the transmission and fluorescence detectors at all times. Thus, it is the configuration of the lock-in amplifier that determines whether circular dichroism or fluorescence polarization anisotropy is recorded. Adding a second lock-in amplifier makes possible simultaneous measurement of circular dichroism and fluorescence polarization anisotropy.

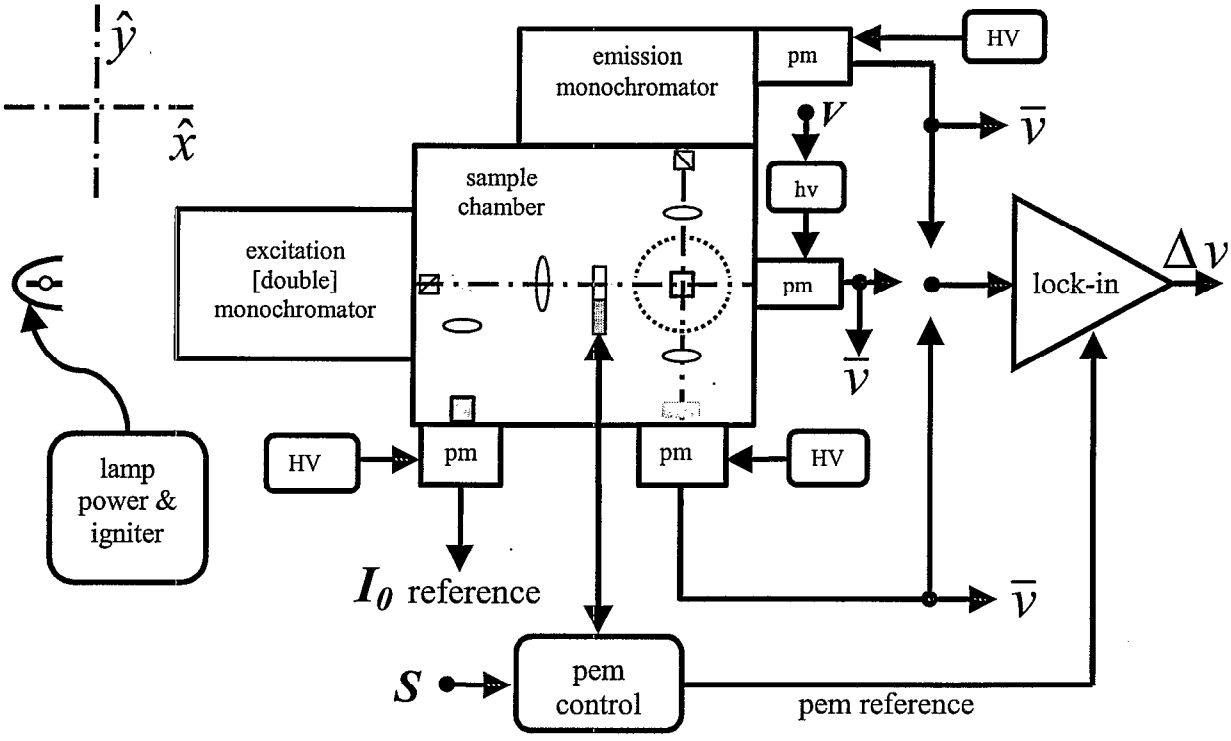

Figure 5 Plan view of an instrument to record circular dichroism or fluorescence polarization anisotropy and pseudo-absorption plus excitation corrected fluorescence at the same time. The excitation source is shown as a high-pressure Xenon arc, although other sources such as a synchrotron radiation storage ring could be used. Optical signals recorded by the various detectors are converted to voltages, $v(t)$, from which time average and modulated signals can be extracted.

A Lock-in Lovers Delight

The accuracy and sensitivity for measuring the intensity of the incident beam is greatly increased by replacing the transparent plate used for diverting a fraction of the incident beam (see Figure 5) with a rotating sectored mirror, which functions as a mechanical chopper. Such devices are widely used in dual beam spectrophotometers. This arrangement sends one half of the incident beam to the reference detector and permits the use of synchronous detection of this signal. The resulting loss of intensity in the primary signal channel can be overcome in part by double synchronous detection, as shown schematically in Figure 6. The "dc" signal is shifted to the frequency of the chopper, hence reducing drift and improving the sensitivity of detection by the use of a lock-in amplifier to record the $\overline{v}$ signal. The Δv signal can be

detected by two lock-ins acting in series. The first is tuned to the frequency of the photoelastic modulator (ω or 2ω, depending on the experiment) but its output filter roll off frequency is set to pass the frequency of the chopper, typically about 100 Hz. This combination increases the selectivity of the detection system, thus compensating for the loss of intensity reaching the detector. Similar configurations have been used for near infrared circular dichroism.[12,13] The significant disadvantage of such a configuration of this approach is an increase in cost, as a complete system for simultaneous measurement of circular dichroism, fluorescence polarization anisotropy, pseudo-absorption, and intensity corrected fluorescence requires seven lock-in amplifiers.

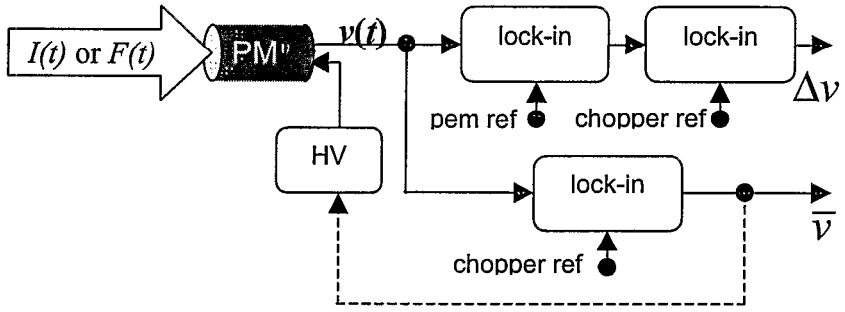

Figure 6 Schematic diagram of the configuration of a double synchronous detection system for the measurement of circular dichroism or fluorescence polarization anisotropy with both a photoelastic modulator and a mechanical mirror chopper.

Polarization Ratio

It is generally preferable to measure the fluorescence polarization anisotropy, r, rather than the polarization ratio, P, as defined in Figure 1 because the former reflects the true weighting of three-dimensional fluorescence.[7] This is fortunate because the approach described for measuring r cannot be adapted to measure P directly. The difficulty arises because measuring P necessitates weighting the parallel and perpendicularly polarized components equally. This requires that J_0 equal 1 or –1 for vertical and horizontally polarized incident radiation, respectively. The first condition can only be achieved for $\delta_0 = 0$, which causes both J_1 and J_2 to equal 0 thus eliminating the polarization difference signals. There is no phase amplitude that causes J_0 to equal –1, as shown in Figure 3.

ACKNOWLEDGMENTS

Research sponsored by the Office of Biological and Environmental Research, United States Department of Energy. I thank John G. Trunk and Betsy M. Sutherland for their comments on the manuscript, and Dr. Yves Dupont, Laboratoire de Biophysique Moléculaire et Cellulaire, CEN, Grenoble, France, for making available the text of a paper prior to its publication.

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