

Time-Resolved Fluorescence Anisotropy Measurements Made Simple

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Time-resolved anisotropy measurements were performed using simple instrumentation with the aim to demonstrate the speed and ease of the experiment. Subsequent data analysis examples involved common, easily adaptable procedures.

KEY WORDS: Time-resolved; anisotropy; polarization; light-emitting diode (LED); time-correlated single photon counting (TCSPC).

INTRODUCTION

The utility and power of time-resolved fluorescence techniques in comparison with steady-state methods are increasingly recognized, thanks to the availability of compact, versatile, and easy-to-use time-correlated single photon counting (TCSPC) spectrometers [1]. These devices [2,3] confute the widespread belief that the necessary instrumentation is complex and difficult to operate. Complicated and very expensive TCSPC electronics built from nuclear instrumentation modules (NIM) can now be integrated on a single personal computer board [4] and controlled by mouse-clicks. Cumbersome mode-locked lasers and flashlamps were gradually replaced by picosecond laser diodes [2] and pulsed light-emitting diodes (LEDs) [3] that practically require no maintenance. Small photosensors or avalanche photodiodes supersede bulky photomultiplier tubes. These achievements changed time-correlated single photon counting from an esoteric research tool for photo-physicists in the 1980s, into a routine laboratory method with many applications in, for example, life sciences.

In comparison with ordinary fluorescence intensity measurements—regardless whether steady-state or time-resolved—polarization (anisotropy) studies are much less

common. This is unfortunate because adding two polarizing elements to an existing spectrometer is relatively easy and opens fascinating possibilities to study molecular orientation and mobility as well as processes that affect them. Potential applications involve investigations of membrane fluidity and rigidity, local microviscosity in liquids and polymers, protein–protein interactions, receptor–ligand binding, and myosin reorientation, all at the molecular level.

The purpose of this article is not to introduce new polarization-based methods or assays, but merely to demonstrate the ease and simplicity of time-resolved anisotropy measurements using inexpensive instrumentation.

BASIC PRINCIPLE

Consider a diluted, liquid solution of a fluorophore. When a pulse of linearly polarized light of appropriate wavelength passes through this sample, fluorophores with their absorption transition vectors aligned parallel to the polarization plane of the light are preferentially excited. Those with vectors perpendicular are not excited at all. This leads to a biased population of excited molecules, which tend to relax to a randomly oriented ensemble because of Brownian rotational diffusion. Simultaneously, the initial excited population decays to the electronic ground state by fluorescence and other processes. The

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polarization plane of the fluorescence photon is determined by the actual orientation of the molecule at the moment of emission: it is therefore well defined shortly after the onset of excitation, but becomes increasingly random as time proceeds. The anisotropy, r , is related to the extent of this randomization and $r(t)$ is the kinetics of the process. Fluorescence anisotropy kinetics cannot be recorded directly, but can be extracted from the decays of polarized emission components.

EXAMPLE ANALYSES

In the following paragraphs, a brief compendium of basic definitions and relations will be given, using real, experimental data as examples. Rigorous treatise of the fluorescence anisotropy can be found in the literature [5,6]. Throughout the text, V stands for vertical and H stands for horizontal polarization with respect to the plane of the spectrometer optics.

Direct Calculation of $r(t)$ from Raw Data

The simplest approach to calculate $r(t)$ is to use the definition of anisotropy literally:

$$r(t) = \frac{VV(t) - VH(t)}{VV(t) + 2 \cdot VH(t)} \quad (1)$$

Here $VV(t)$ and $VH(t)$ denote fluorescence decay kinetics of the sample measured using V polarized exciting light and detecting the emission components polarized V and H, respectively. $VV(t)$ and $VH(t)$ are often referred as parallel and perpendicular polarized decays. This procedure assumes that $VV(t)$ and $VH(t)$ are recorded under identical experimental conditions (except for the emission polarizer setting, of course). In practice, it means constant excitation intensity, equal acquisition times, and equal detection efficiency for V and H polarized components. The latter requirement is difficult to fulfill in general; therefore, the definition of $r(t)$ is often written as

$$r(t) = \frac{VV(t) - G \cdot VH(t)}{VV(t) + 2 \cdot G \cdot VH(t)} \quad (2)$$

where G is an instrument and wavelength-dependent correction factor to compensate for the polarization bias of the detection system. The precise G -factor can be determined either by so-called tail matching of $VV(t)$ and $VH(t)$ decays, or by treating the additionally measured $HV(t)$ and $HH(t)$ curves. The latter procedure is always applicable, but can be time consuming because it involves two additional decay measurements with the excitation polarizer set to H. Then, $G = \int HV(t) dt / \int HH(t) dt$.

Application of tail matching assumes some prior knowledge about the system under study: the anisotropy decay must be faster than the intensity decay and the rotational diffusion must be unhindered.

The filter-based detection optics is nearly ideal for anisotropy investigations, i.e., $G \approx 1$. To demonstrate this, a dilute solution (absorbance = 0.03, approximately 6×10^{-7} M) of Coumarin 6 in spectral-grade ethylene glycol was prepared and measured at 25°C, using a FluoroTime 100 spectrometer in connection with a TimeHarp 200 TCSPC board (PicoQuant, Berlin, Germany). The sample was excited by 680 ps (full width at half-maximum) pulses of a PLS 450 LED source driven by PDL800-B driver with 10 MHz repetition rate (PicoQuant). Fluorescence with wavelengths $\lambda_{Em} > 530$ nm was selected by a long-pass filter. Inexpensive sheet polarizers were used to V polarize the LED radiation, as well as to select the V- or H-polarized emission component of the fluorescence. The relevant spectral conditions are depicted on Fig. 1. As illustrated, polarizers reduce both the excitation and collected emission intensity roughly by a factor of 3.

Thus, in comparison to simple lifetime measurement, which can be performed without polarizers, only approximately 10% of the signal intensity is available now. In addition to that, anisotropy investigations require decay data measured with very high statistical precision because calculation of $r(t)$ involves division of a small difference by a large sum. To make the difference term significant, a large number of counts should be collected. Thanks to a high repetition rate excitation source and efficient collection optics, the decay curves presented on Fig. 2 were recorded very quickly: 20 s each. $VV(t)$ and $VH(t)$ clearly

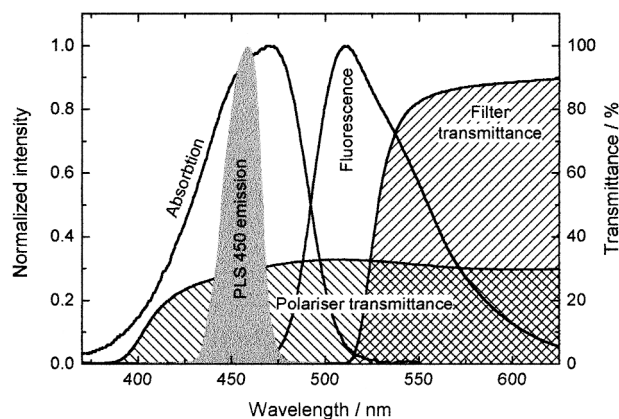


Fig. 1. Absorption and fluorescence emission spectra of Coumarin 6 dissolved in ethylene glycol. The emission spectrum of PLS 450 pulsed-LED diode used as excitation source is also included. The right axis belongs to the long-pass filter's and sheet polarizer's transmission spectra (shaded areas).

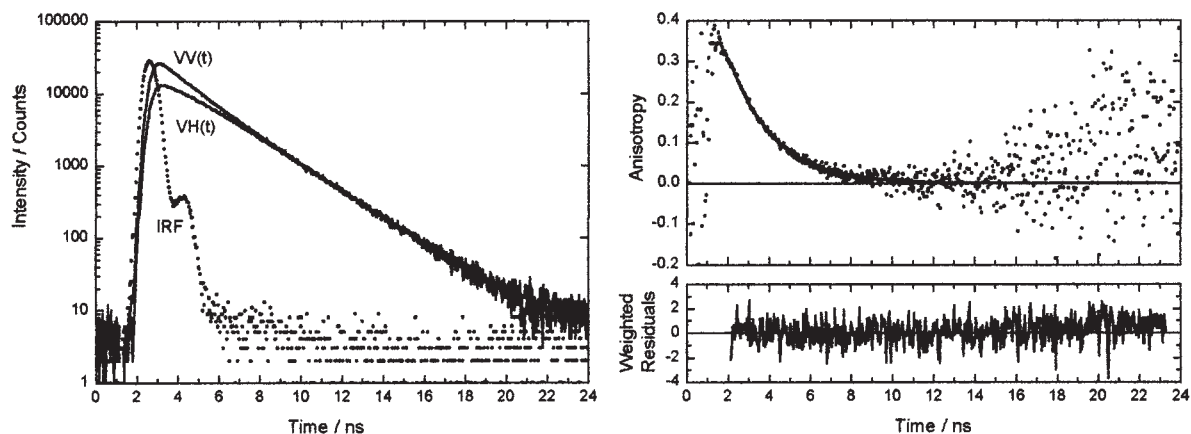


Fig. 2. VV and VH polarized decays of Coumarin 6 in ethylene glycol and the instrument response function, assuming $G = 1$, is plotted at right. An appropriate portion of $r(t)$ was fitted to a single-exponential decay model without reconvolution. The fitted curve and the properly weighted residuals are also shown at the right panel.

differ during the first few nanoseconds, but then decay in a similar manner. The obvious reason is the finite rotational speed of Coumarin molecules in a viscous solvent. The anisotropy decay curve as calculated by FluoFit software (PicoQuant), using $G = 1$, is plotted at right.

An advantage of this simple and quick approach is the immediate visual check of the resulting $r(t)$. Indeed, many biologically relevant problems (e.g., ligand binding or diffusion of fluorophore embedded in membrane) can be solved simply by comparison of the $r(t)$ shape of different samples. However, numerical evaluation is still possible and useful. Figure 2 shows that $r(t)$ decays to zero within ≈ 8 ns after the onset of excitation; in other words, the residual anisotropy r_∞ is zero. This means that the rotation is unrestricted and the rotational relaxation is finished before the complete decay of fluorescence. Furthermore, one can roughly estimate the fundamental (or initial) anisotropy, $r(t = 0) \equiv r_0$. Most of fluorescence investigations employ mono-photon $S_0 \rightarrow S_n$ ($n = 1, 2$) excitation and detect the $S_1 \rightarrow S_0$ emission, like this study. For photoselection in isotropic solution, r_0 is determined by the angle γ between absorption and emission transition moment:

$$r_0 = (3 \cos^2 \gamma - 1)/5 \quad (3)$$

For $S_0 \rightarrow S_1$ absorption and $S_1 \rightarrow S_0$ fluorescence, these transition moment vectors are often close to parallel, and therefore $r_0 \approx 0.4$. Anisotropy decay of a fluorophore with $\gamma = 90^\circ$ displays an increase of r from -0.2 to zero, whereas for a molecule with $\gamma \approx 54.7^\circ$, the $r(t)$ is constantly zero, regardless of the photoselection and subsequent rotation. In our case, the time-zero position is uncertain because of the relatively broad instrument response function (IRF), but $r \approx 0.3$ where the IRF is peaking. This anisotropy value would be measured by

steady-state fluorimeter for Coumarin 6 in vitrified solution, for example, in ethylene glycol cooled to -10°C .

The decay kinetics of $r(t)$ is also informative. It can be shown [8] that rotational diffusion of a spherical particle leads to exponential anisotropy decay characterized by an initial anisotropy value and a rotational correlation time τ_r .

$$r(t) = r_0 \cdot \exp(-t/\tau_r) \quad (4)$$

The shape of a real molecule can be reasonably approximated by a suitable ellipsoid of revolution. Then $r(t)$ has a multiexponential form. The quality of experimental data rarely allow one to resolve more than two rotational components, and in the majority of cases the spherical rotor description is sufficient. Hence, one can try to fit a single exponential decay model to the calculated $r(t)$ to yield the anisotropy parameters, bearing in mind that anisotropy is a ratio function: reconvolution fitting procedure (common for intensity decay analysis) cannot be applied and a different data-weighting scheme should be used [5, 6]. Figure 2 shows an example. The fit yields $\tau_r = 2.1 \pm 0.1$ ns, and $r_\infty = 0.000 \pm 0.001$. The initial anisotropy value r_0 determined in this way is not reliable because it strongly depends on the selected left limit of the fitting range.

Reconvolution Analysis of Polarized Decays

Recording the time-evolution of fluorescence intensity with a fixed polarization plane means observing a superposition of rotational diffusion and excited-state population decay. This makes the $VV(t)$ and $VH(t)$ of the same sample different. Fortunately, these processes can be separated by suitable data analysis. Let us denote $F(t)$ the

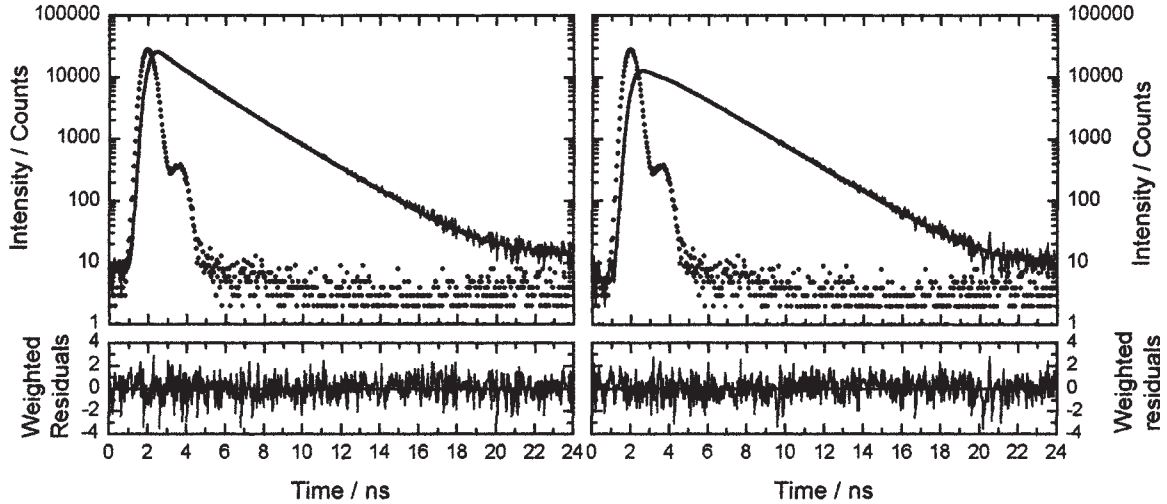


Fig. 3. Double-exponential reconvolution analyses of VV (at left) and VH (at right) polarized decays of Coumarin 6 in ethylene glycol.

pure excited-state population decay, free of anisotropy effects. It can be shown that

$$\begin{aligned} VV(t) &= [1 + 2r(t)] \cdot F(t) \\ VH(t) &= [1 - r(t)] \cdot F(t) \end{aligned} \quad (5)$$

In anisotropy studies, it is advantageous to use a fluorophore with single lifetime τ_F , because data analysis is then easier and we can write $F(t) = F_0 \cdot \exp(-t/\tau_F)$. Substituting Eq.(4) and $F(t)$ into Eq.(5) yields

$$\begin{aligned} VV(t) &= F_0 \left[2r_0 \exp\left(-t \frac{\tau_F \tau_r}{\tau_F + \tau_r}\right) + \exp(-t/\tau_F) \right] \\ VH(t) &= F_0 \left[-r_0 \exp\left(-t \frac{\tau_F \tau_r}{\tau_F + \tau_r}\right) + \exp(-t/\tau_F) \right] \end{aligned} \quad (6)$$

Both $VV(t)$ and $VH(t)$ decays obey double-exponential kinetics; contain information on r_0 , τ_r and τ_F ; and can be analyzed using ordinary reconvolution fitting routines. G -factor correction is not necessary, but one needs appropriate IRFs. (Usually, the instrument response does not depend on polarizer settings and it is recorded as VV . In certain cases, different IRF^{VV} and IRF^{VH} might be necessary.) In theory, r_0 , τ_r , and τ_F can be recovered by fitting any of the two polarized decays. In practice, it is much more reliable to analyze both $VV(t)$ and $VH(t)$ with common fixed τ_F . The fluorescence lifetime should be available from independent measurements performed without polarizers, or can be determined by fitting the decay recorded with the emission polarizer set to so-called magic angle, i.e. $\approx 54.7^\circ$, denoted M hereafter.

To demonstrate this method, we reanalyze the decays from Fig. 2. The fluorescence lifetime of Coumarin 6 in ethylene glycol was determined by single-exponential re-

convolution fitting of the M-polarized decay $VM(t)$. The recovered lifetime is 2.31 ns with normalized chi square value, $\chi^2 = 1.12$. Double-exponential analyses of $VV(t)$ and $VH(t)$ with fixed $\tau_2 = \tau_F = 2.31$ ns yield two amplitudes (A_1 and A_2) and the second decay or rise time τ_1 . According to Eq. (6), $r_0 = A_1/2A_2$ from VV results or $r_0 = -A_1/A_2$ from VH results; $\tau_r = \tau_1 \tau_2 / (\tau_2 - \tau_1)$ from both decays. The fits are shown on Fig. 3 and the results are summarized in Table I.

The recovered anisotropy parameters are consistent with the results from direct calculation of $r(t)$. A slight mismatch between the r_0 and τ_r values calculated from differently polarized decays is due to the correlation of freely varying model parameters. According to Eq. (6), the two amplitudes are not independent, but linked through the value of r_0 . Global fitting of $VV(t)$ and $VH(t)$ with appropriate linked lifetimes and amplitudes gives the best results in this case, but this goes beyond the scope of this article.

The power of this method becomes more obvious as τ_r decreases. For example, Coumarin 6 rotates in a less viscous dimethyl sulfoxide much faster. The spectral conditions are practically the same as depicted on Fig. 1 and

Table I. Summary of Fit Results for Coumarin 6 in Ethylene Glycol

Polarization	τ_1 (ns)	A_1	A_2	χ^2	r_0	τ_r (ns)
VV	1.02	14,200	23,900	1.08	0.30	2.10
VH	1.09	-8200	24,000	1.04	0.34	2.05
Average values:					0.32	2.08

A_1 , τ_1 , amplitude and lifetime of the fast decay component related to molecular rotation, A_2 , amplitude of the main decay component; r_0 , τ_r , calculated initial anisotropy and rotational correlation time, χ^2 normalized chi square value of the fit.

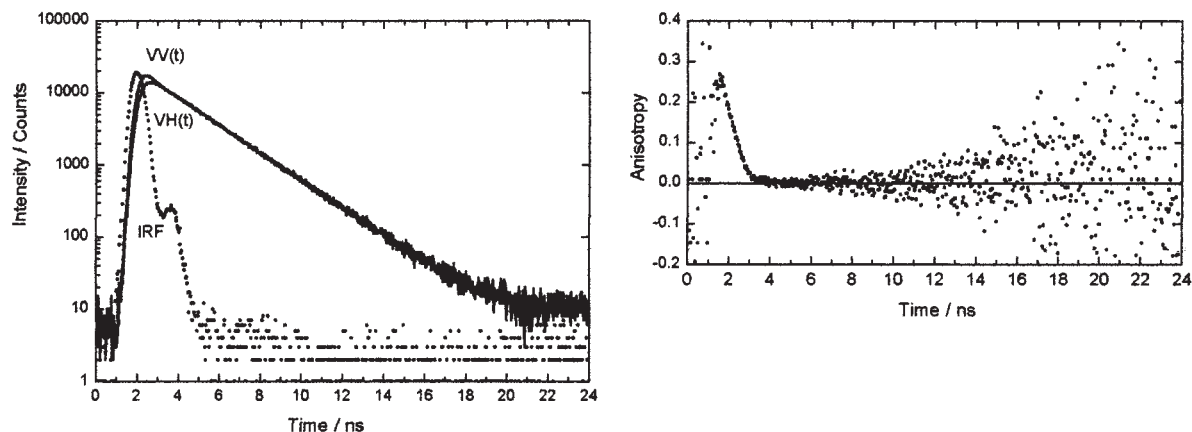


Fig. 4. VV and VH polarized decays of Coumarin 6 in dimethyl sulfoxide and the instrument response function. The anisotropy decay curves calculated from $VV(t)$ and $VH(t)$ directly, is plotted at right panel.

the fluorescence lifetime is also similar. Analysis of $VM(t)$ gives $\tau_F = 2.23$ ns with $\chi^2 = 1.15$. Polarized decays on Fig. 4 reveal that the depolarization is finished within the duration of the 680 ps wide excitation pulse. Direct analysis of the $r(t)$ curve shown at right would be meaningless, but reconvolution fitting of $VV(t)$ and $VH(t)$ gives reasonable results which are summarized in Table II.

The larger spread (or, in other words, the larger uncertainty) of the recovered anisotropy parameters is a consequence of the rapid rotation together with parameter correlation mentioned above. τ_r is shorter than the excitation pulse duration, and the resulting short decay components associated with $r(t)$ are difficult to resolve. However, we still have two datasets and a constrained data analysis model. The average values are still reliable.

Table II. Summary of Fit Results for Coumarin 6 in Dimethyl Sulfoxide

Polarization	τ_f (ns)	A_1	A_2	χ^2	r_0	τ_r (ns)
VV	0.30	10,200	19,900	1.18	0.26	0.34
VH	0.25	-6700	20,400	1.14	0.33	0.28
Average values:					0.30	0.31

A_1 , τ_f , amplitude and lifetime of the fast decay component related to molecular rotation, A_2 , amplitude of the main decay component, r_0 , τ_r , calculated initial anisotropy and rotational correlation time, χ^2 normalized chi square value of the fit.

SUMMARY AND CONCLUSIONS

Quick example anisotropy studies were conducted using simple-to-use hardware. Stable, high repetition rate sub-nanosecond LED excitation sources in connection with highly efficient filter-based detection optics more than compensate for the signal intensity loss caused by the polarizers. This allowed for data acquisition times as low as few tenths of seconds, without polarizer toggling or simultaneous V and H detection (T-geometry). Two data treatment techniques were demonstrated. Both can be easily adopted because they do not use any advanced fitting features, such as global analysis.

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