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Analysis of complex anisotropy decays from single-frequency polarized-phasor ellipse plots

Noga Kozer and Andrew H A Clayton

Abstract

The anisotropy decay of a fluorescently-labelled macromolecule provides information on the internal and global dynamics of the macromolecule. Weber was a pioneer of fluorescent probes, polarization and polarized phase-modulation methods and revealed the power of combining or comparing these methods to disentangle complex modes of emission depolarization. In this paper we take a similar course and show that when measurements of dynamic depolarization are combined with steady-state anisotropy, complex anisotropy decays can be deduced from measurements at a single modulation frequency. Specifically, a double exponential anisotropy decay can be resolved by combining one of the polarized emission phasors with the steady-state anisotropy. The key is the polarized phasor ellipse plot which provides a convenient visualisation aid and reduces the dimensionality of the minimisation problem from three variables to one variable. We illustrate these concepts with an experimental measurement of the anisotropy decay of a small cytoplasmic fluorescent probe in live cells.

1. Introduction

The emission anisotropy (or polarization) continues to be an important quantity in biological fluorescence since the pioneering studies of Weber [1]. Weber realised that proteins are not static but highly dynamic ‘kicking and screaming’ entities and he pioneered the development of fluorescence methods and probes to study these dynamics [2]. Fluorescent probes that are attached to biological macromolecules can potentially provide useful information about local motional dynamics at or near the point of probe attachment and about global dynamics of the macromolecule. Measurement of the ensuing fluorescence dynamics can be performed in either the time-domain (anisotropy decay) [3] or frequency-domain (dynamic depolarization) [4].

Generally speaking, complex anisotropy decay models require high quality time-resolved data [5] or phase and amplitude data recorded at several modulation frequencies [6]. This circumstance is challenging to achieve in live cell environments where prolonged acquisition times can lead to reduction in signal due to photo-bleaching, compromised cell vitality owing to photo-toxicity, and inability to track biological processes in real-time. Instead, wide-field time-gating (in the time-domain) [7] or wide-field single-frequency approaches (in the frequency domain) [8] provide spatial information in parallel with the required reduced acquisition times to capture biologically-relevant phenomena and reduced light dose. However, the resolution of these measurements is compromised owing to the limited number of photons collected, limited number of time-gates or limited number of modulation frequencies employed. This raises the question, what useful information can be extracted from single time-gate or single-frequency polarized fluorescence measurements?

The classical studies of Weber and others revealed the power of combining steady-state polarization measurements with phase measurements at a single modulation frequency [9–11]. For example, Weber showed that both the rate of rotation and the limiting initial polarization could be obtained by measuring lifetimes of the parallel or perpendicular polarized components of the emission and comparing these values to magic angle conditions [4]. Weber also showed that in the case of anisotropic rotations which occur in rotators of non-spherical symmetry, estimates of the rotational correlation times and ratios of principal axes of rotations could...
be obtained from differential phase measurements and steady-state polarization [9]. Lakowicz extended these ideas to highly anisotropic, hindered rotations of probes in bilayers and revealed that estimates of rotation rates and degree of angular hindrance could be obtained by combining differential phase measurements with steady-state polarization [10]. Weber also dealt with the case where changes in fluorescence polarization occur in a complex cellular environment in response to a biochemical perturbation. If only the polarization information was used alone then estimates of fractional population change and rotational rate of the changing population varied widely. However, by combining the polarization information with differential phase information, he was able to decrease the range of fractional population changes and rotational rates [11]. The Weber formalism was also used more recently in wide-field frequency-domain dynamic depolarization to get estimates of apparent correlation times and limiting anisotropies in a wide-field image by combining the differential phase measurement image with the steady-state polarization image [8].

The phasor or polar plot was first described for frequency-domain fluorescence measurements by Jameson and co-workers and has found great utility in conventional fluorescence lifetime imaging microscopy in both the frequency-domain and time-domain [12–15]. In recent work, the polar plot was extended for the display and analysis of single frequency frequency-domain dynamic depolarization experiments [16]. In this analysis the parallel and perpendicular polarized components of the emission are recorded and the data presented on a polar plot. Thus for a detected modulation of $m$ and phase $\varphi$, $x = m \cos \varphi$ and $y = m \sin \varphi$ and there are two points on the polar plot representing the parallel and perpendicular-polarized components of the emission. For single correlation time models or single correlation time/hindered rotation models the rotational correlation time and the excited state lifetime can be obtained using this approach by essentially fitting the parallel and perpendicular components of the phasor to a straight-line [16]. The advantage of this method is that the lifetime, steady-state anisotropy and initial limiting anisotropy do not need to be measured. However an obvious limitation is that only very simple anisotropy decay models can be analysed [16] and the information content does not extend beyond previous formalisms.

The purpose of the present paper is to demonstrate that when the initial anisotropy, excited state lifetime and steady-state anisotropy are known, as in the earlier studies of Weber, then a two correlation time model can be extracted from measurement of either the parallel-polarized phasor or the perpendicular-polarized phasor at a single modulation frequency.

The paper is organised as follows. In the first section we review the theory of the phasor plot, the theory of the polarized phasor plot and extend to the case of two rotational correlation times. We then apply the analysis to data collected from live cells stained with fluorescein diacetate. Our single-frequency measurement and analysis reveals the inadequacy of the single correlation time model to describe the rotational dynamics of this probe in the cellular milieu. Instead the new and improved analysis indicates the presence of short and long rotational correlation time probe motions, consistent with published multi-frequency analysis.

### 1.1. Summary of experimental parameters and theoretical models

The theory section attempts to present the mathematical equations used to analyse single-frequency polarization experiments in terms of the phasor approach. We cover increasingly complex fluorescence systems from single correlation time, single lifetime, through to double exponential lifetime and double exponential correlation time. Because the information available to the experimentalist can vary widely, we provide a summary table as a guide to the analysis of polarization experiments. Table 1 contains different combinations of experimental inputs, the type of model that can be analysed with those inputs and a reference to

| Exp | Exp | Exp | Exp | Exp | Exp | Equation | Theory | Theory | Theory |
|-----|-----|-----|-----|-----|-----|----------|--------|--------|--------|
| $t_0$ | $r$ | $\tau$ | $\alpha$, $\tau_1$, $\tau_2$ | $\Phi_{par}$ | $\Phi_{perp}$ | (1) and (2) | $\phi$ | $\tau$ | $\phi_1$, $\phi_2$, $\beta$
| Yes | Yes | Yes | No | No | No | $\tau_1$ | Yes | Given | No |
| Yes | No | Yes | No | No | Yes | $\tau_2$ | Yes | Given | No |
| Yes | No | Yes | No | No | Yes | $\Phi_{par}$ | Yes | Given | No |
| No | No | No | No | Yes | Yes | $\Phi_{perp}$ | Yes | Given | Yes |
| Yes | Yes | No | No | Yes | No | $\Phi_{par}$ | Yes | Given | No |
| Yes | No | No | No | Yes | Yes | $\Phi_{perp}$ | Yes | Given | Yes |
| Yes | Yes | No | Yes | Yes | No | $\tau_1$ | Yes | Given | No |
| Yes | Yes | No | Yes | Yes | No | $\tau_2$ | Yes | Given | No |
| Yes | No | No | No | Yes | Yes | $\Phi_{par}$ | Yes | Given | Yes |
| Yes | Yes | No | Yes | Yes | No | $\Phi_{perp}$ | Yes | Given | Yes |

Note: In the exp column a yes means that a parameter(s) is measured, while in the theory column a yes means that the value of the parameter(s) can be determined using the equations contained in the equation column.
the equation used to analyse those inputs in the theory section.

2. Theory

2.1. Steady-state anisotropy

Although our paper is focussed on dynamic determinations of rotation, for completeness, we include the steady-state determination of the rotational correlation time using the fluorescence anisotropy.

The steady-state anisotropy for an isotropic rotator is given by

\[ r = r_0(1 + (r \phi)). \] (1)

And the rotational correlation time,

\[ \phi = \tau r/(r_0 - r) \] (2)

where \( \phi \) is the rotational correlation time, \( \tau \) is the fluorescence lifetime, \( r \) is the steady-state anisotropy and \( r_0 \) is the limiting anisotropy (in the absence of rotation, i.e. usually measured in a vitrified solution).

2.2. Time-resolved fluorescence in the frequency-domain

We begin by recalling that both frequency-domain and time-domain lifetime experiments can be represented on a 2D plot called the phasor or polar plot [12–15].

The output from a frequency domain experiment is the phase and modulation of the fluorescence which is represented on the polar plot by a vector of length \( m \), subtended by an angle \( \varphi \). The horizontal component of the vector is then \( x = m \cos \varphi \) and the vertical component of the vector is \( y = m \sin \varphi \).

For single component systems i.e. those systems governed by a single exponential decay in time, the cosine component of the phasor and the sine component of the phasor are given by:

\[ I(t) = \exp(-t/\tau) \]

\[ X = m \cos \varphi = 1/(1 + (\omega \tau)^2) \] (4)

\[ Y = m \sin \varphi = \omega \tau/(1 + (\omega \tau)^2). \] (5)

A plot of \( x \) versus \( y \) for single component systems traces out a semi-circle, sometimes referred to as the universal circle. A single exponentially decaying population of fluorophores will be represented as a phasor on the universal circle with a position that specifies the lifetime of the fluorescent species.

For the sum of two exponential-decaying components with lifetimes \( \tau_1 \) and \( \tau_2 \), the total cosine and sine components of the phasor are given by the weighted sum of the components. In other words the phasors can be added in the same way as vectors, thus simplifying the representation of complex mixtures of fluorescence.

\[ m \cos \varphi = \alpha m \cos \varphi_1 + (1 - \alpha)m \cos \varphi_2 \]

\[ = \alpha/(1 + (\omega \tau_1)^2) + (1 - \alpha)/(1 + (\omega \tau_2)^2) \] (6)

\[ m \sin \varphi = \alpha m \sin \varphi_1 + (1 - \alpha)m \sin \varphi_2 \]

\[ = \alpha \omega \tau_1/(1 + (\omega \tau_1)^2) + (1 - \alpha)\omega \tau_2/(1 + (\omega \tau_2)^2). \] (7)

Mixing between two components (i.e. \( \alpha \) variable and \( \tau_1 \) and \( \tau_2 \) fixed) can be represented on the polar plot as a chord which intersects the semi-circle at phasor positions corresponding to the two lifetime components, figure 1. The position of the experimental phasor along the chord is related to the fractional fluorescence contribution of each lifetime to the total decay.

Figure 1. Polar plot representation of time-resolved fluorescence in the frequency-domain. Semi-circle indicates phasor positions of single exponential decaying fluorescence species. The solid dots denote phasor positions for lifetimes \( \tau_1 \) and \( \tau_2 \). The chord represents the mixture of two exponential decays with \( \tau_1 \) and \( \tau_2 \). The position of the point on the chord denotes the fraction fluorescence contribution of each lifetime to the total decay.

2.3. Polarized fluorescence in the frequency-domain

2.3.1. Single correlation time models

2.3.1.1. One lifetime \( \tau \) and one rotational correlation time \( \phi \)

The polarized components of the emission are conventionally observed through linear polarizers that are set parallel to the excitation polarization direction and perpendicular to the excitation polarization direction. For an anisotropy decay denoted by \( r(t) \) and an intensity decay denoted by \( I(t) \), the expressions for the parallel and perpendicular components of the emission are:

\[ I_{\parallel} = (1 + 2r(t))I(t) \] (8)

\[ I_{\perp} = (1 - r(t))I(t). \] (9)

For a simple anisotropy decay with single correlation time \( \phi \) and limiting initial anisotropy of \( r_0 \), the time-resolved anisotropy decay is given by,

\[ r(t) = r_0 \exp(-t/\phi). \] (10)

Combining equations (8)–(10), we have the following expressions for the parallel-polarised component of the emission and the perpendicular-polarised component of the emission:

\[ I_{\parallel} = \exp(-t/\tau) + 2r_0 \exp(-t/\phi) * \exp(-t/\tau) \]

\[ = \exp(-t/\tau) + 2r_0 \exp(-t/T_2). \] (11)
\[ I_{\text{perp}} = \exp(-t/\tau) - r_0 \exp(-t/\phi) \ast \exp(-t/T_2) \]
\[ = \exp(-t/\tau) - r_0 \exp(-t/T_2) \]  
where \( T_2 = \tau/(1 + \tau/\phi) \).

Thus the parallel-polarized emission for single correlation time, single lifetime model appears as a double exponential decay in time. The perpendicular-polarized emission has the same time constants as the parallel-polarized emission but has negative amplitude in the second exponential term.

In the frequency-domain we can use expressions (6) and (7) to represent the double-exponential decay processes provided that we note that \( \tau \) is the excited state lifetime, \( T_2 \) is a function of the lifetime and the rotational correlation time and \( \phi \) is the fractional fluorescence contribution of the lifetime and \( \phi \) also depends on whether the emission is detected in the parallel or perpendicular polariser orientation. More explicitly the cosine and sine components of the phasor are given by the expressions,

\[ m \cos \varphi(\text{par}) = \alpha_{\text{par}} m \cos \varphi_1 + (1 - \alpha_{\text{par}}) m \cos \varphi_2 \]
\[ = (2r_0 T_2 / 2r_0 T_2 + \tau)(1 + (\omega T_2)^2) \]
\[ + (\tau / 2r_0 T_2 + \tau)(1 + (\omega T_2)^2) \]  
(13)

\[ m \sin \varphi(\text{par}) = \alpha_{\text{par}} m \sin \varphi_1 + (1 - \alpha_{\text{par}}) m \sin \varphi_2 \]
\[ = (2r_0 T_2 / 2r_0 T_2 + \tau) \omega \tau / (1 + (\omega T_2)^2) \]
\[ + (\tau / 2r_0 T_2 + \tau) \omega \tau / (1 + (\omega T_2)^2) \]  
(14)

\[ m \cos \varphi(\text{perp}) = \alpha_{\text{perp}} m \cos \varphi_1 + (1 - \alpha_{\text{perp}}) m \cos \varphi_2 \]
\[ = (r_0 T_2 / r_0 T_2 - \tau)(1 + (\omega T_2)^2) \]
\[ + (-\tau / r_0 T_2 - \tau)(1 + (\omega T_2)^2) \]  
(15)

\[ m \sin \varphi(\text{perp}) = \alpha_{\text{perp}} m \sin \varphi_1 + (1 - \alpha_{\text{perp}}) m \sin \varphi_2 \]
\[ = (r_0 T_2 / r_0 T_2 - \tau) \omega \tau / (1 + (\omega T_2)^2) \]
\[ + (-\tau / r_0 T_2 - \tau) \omega \tau / (1 + (\omega T_2)^2) \]  
(16)

We denote the \( x, y \) position of the polarized phasor on the phasor plot as

\[ \text{Ph}_{\text{par}}(M \cos \varphi(\text{par}), M \sin \varphi(\text{par})) = \text{Ph}_{\text{par}}(r_0, \tau, \phi) \]  
(17)

\[ \text{Ph}_{\text{perp}}(M \cos \varphi(\text{perp}), M \sin \varphi(\text{perp})) = \text{Ph}_{\text{perp}}(r_0, \tau, \phi) \]  
(18)

When plotted with a variable rotational correlation time (with lifetime and limiting anisotropies fixed), the polarized-phasors trace out a trajectory that resembles a pair of Cassinian ellipses.

As an example, the phasor trajectories corresponding to parallel and perpendicular components of the emission are sketched in figure 2 for the case \( r_0 = 0.4, \tau = 4 \text{ ns}, \phi = 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 \text{ ns} \) (see also [16]).

For the case relevant to this paper (i.e. when initial anisotropy, steady-state anisotropy and lifetime are known), then the value of the rotational correlation time can be deduced by plotting one of the experimental polarized phasors on the polarized phasor ellipse plot. An isotropic rotator will have the corresponding polarized phasor on the polarized phasor ellipse and at a location that specifies the value of the rotational correlation time. In contrast, a non-isotropic rotator will have a polarized phasor position which is inside the polarized phasor ellipse plot.

2.3.1.2. One lifetime \( \tau \) and one hindered-rotational correlation time \( \phi \) A special case of a non-isotropic rotator with one correlation time is the hindered rotator. This case was discussed previously and we simply state the result here. From measurements of the parallel and perpendicular components of the phasor one essentially draws a straight line though the two phasors. The gradient of the straight line is given as grad and the intercept as int,

\[ \text{Grad} = [m \sin \varphi(\text{par}) - m \sin \varphi(\text{perp})]/ 
\]
\[ m \cos \varphi(\text{par}) - m \cos \varphi(\text{perp}) \]  
(19)

\[ \text{Int} = m \sin \varphi(\text{par}) - \text{Grad} \times m \cos \varphi(\text{par}) \]  
(20)

The lifetime and correlation time can be obtained as solutions of the quadratic equation,

\[ T_{1,2} = (1 \pm (1 - 4 \text{int(\text{grad}^2)/2 \text{int}})^{1/2})/2 \text{int} \omega \]  
(21)

where \( T_1 = \tau \) and \( T_2 = \tau/(1 + \tau/\phi) \) is given above.

2.3.1.3. Two lifetimes \( \tau_1 \) and \( \tau_2 \) and one rotational correlation time \( \phi \) The total polarized phasor for an isotropic rotator with two lifetime decays is the weighted sum of the individual polarized phasors
for the lifetime components $\tau_1$ and $\tau_2$ where $\alpha$ is the fractional fluorescence contribution of lifetime $\tau_1$ to the intensity decay (see equations (4) and (5)).

$$
\text{Ph}_{\text{perp}}(\phi) = \alpha \text{Ph}_{\text{perp}}(\tau_0, \tau_1, \phi) + (1 - \alpha) \text{Ph}_{\text{perp}}(\tau_0, \tau_2, \phi).$$

(22)

Here, $\alpha, \tau_1, \tau_2, \tau_0$ are fixed while $\phi$ is varied to generate the polarized phasor ellipse for isotropic rotator with two lifetime components.

For the case relevant to this paper we assume if $\alpha, \tau_1, \tau_2, \tau_0$ are all known then the experimental polarized phasor can be plotted on the polarized phasor ellipse and as above, if the experimental phasor lies on the polarised phasor ellipse (equation (22)) then the value of the rotational correlation time can be determined. In contrast, a non-isotropic rotator will have a polarized phasor position which is inside the polarized phasor ellipse plot.

2.3.2. Double-exponential rotational correlation time models

2.3.2.1. Single lifetime $\tau$ and two correlation times $\phi_1$ and $\phi_2$ The simplest way to introduce double exponential correlation time models is to begin with the polarized phasor for the isotropic rotator. For a given $\tau_0$ and lifetime, and for $0.01 \tau < \phi < 100 \tau$ there is a one to one correspondence between position on the polarized phasor plot and correlation time (see equations (11)–(14)). However, convergence of polarized phasors occurs when $\tau \ll \phi$ and $\tau \gg \phi$. A simulated plot for the perpendicular polarized phasor (with $\tau_0 = 0.4, \tau = 4$ ns) is illustrated in figure 3. Let us denote this polarized phasor by $\text{Ph}(\tau_0, \tau, \phi)$. For a double exponential rotational correlation time model the simplest representation is to add the two polarized phasors corresponding to correlation time 1 and correlation time 2 i.e.

$$
\text{Ph}_{\text{perp}}(\beta, \phi_1, \phi_2) = \beta \text{Ph}_{\text{perp}}(\tau_0, \tau, \phi_1) + (1 - \beta) \text{Ph}_{\text{perp}}(\tau_0, \tau, \phi_2)
$$

(23)

where $\beta$ is the fractional fluorescence contribution of correlation time 1 to the anisotropy decay, and $(1 - \beta)$ is the fractional fluorescence contribution of correlation time 2 to the anisotropy decay. $\text{Ph}_{\text{perp}}(\tau_0, \tau, \phi_1)$ and $\text{Ph}_{\text{perp}}(\tau_0, \tau, \phi_2)$ are given by equations (15)–(18) as above.

Analogously for double exponential intensity decays, a double exponential anisotropy decay phasor will be located inside the polarised phasor ellipse (c/f universal circle) and at a point on the chord that intersects with ellipse at phasor positions for the ‘pure’ correlation times, $\phi_1$ and $\phi_2$. Figure 3 illustrates a chord intersecting phasor positions corresponding to rotational correlation times of 0.1 ns and 20 ns. The position of the point along the chord is proportional to the fractional fluorescence contribution of the 20 ns component.

2.3.2.2. Two lifetimes $\tau_1$ and $\tau_2$ and two rotational correlation times $\phi_1$ and $\phi_2$ (non-associative) The total polarized phasor is weighted-sum of the individual polarized phasors for isotropic rotators with the same lifetime components ($\tau_1, \tau_2, \alpha$).

$$
\text{Ph}(\beta, \phi_1, \phi_2) = \beta \text{Ph}(\tau_0, \tau_1, \phi_1) + (1 - \beta) \text{Ph}(\tau_0, \tau_2, \phi_2)
$$

(24)

Using equation (22), the polarized phasor ellipse corresponding to an isotropic rotator with two lifetimes is generated. For the double exponential correlation time, the experimental polarised phasor will lie inside the polarised phasor ellipse and a chord through the experimental point will indicate the two correlation times.

2.3.2.3. Two lifetimes $\tau_1$ and $\tau_2$ and two rotational correlation times $\phi_1$ and $\phi_2$ (associative) The total polarized phasor for the two lifetime, two rotational correlation time associative case is given by,

$$
\text{Ph}(\beta, \phi_1, \phi_2) = \beta \text{Ph}(\tau_0, \tau_1, \phi_1) + (1 - \beta) \text{Ph}(\tau_0, \tau_2, \phi_2)
$$

(25)

2.4. Steady-state anisotropy as a goodness-of-fit parameter

For all the models with a single correlation time, the steady state anisotropy derived correlation time (equation (2)) can be compared with the correlation time deduced from either equations (19)–(21) or from the position of the experimental phasor on the polarized phasor ellipse (equations (13)–(18) and (22)). Good agreement between correlation time values would imply that the measurements do not demand a more complex model to account for them. On the other hand, if two or more determinations assuming
an isotropic rotator do not yield good agreement then the more complex models are justified.

For all of the models involving two rotational correlation times the polarized phasor lies inside the polarized phasor ellipse. In this circumstance, correlations between parameters are fixed according to geometry and vector algebra. For example once a value of $\phi_1$ is chosen (‘guessed’), the other two parameters $\phi_2$ and $\beta$ are determined by the point of intersection of the line with the polarized phasor ellipse and the relative distance subtended by the experimental phasor relative to the total length of the line. To determine which line fits best with experiment we turn to the steady-state anisotropy as a goodness of fit parameter.

The expression for the steady-state anisotropy for one lifetime, one correlation time is given by equation (1).

The expression for the steady-state anisotropy for one lifetime and two correlation times is given by equation (26).

\[
 r = \beta \tau_0/(1+(\tau/\phi_1)) + (1-\beta) \tau_0/(1+(\tau/\phi_2)).
\]

Once $\phi_1$ is guessed, $\phi_2$ and $\beta$ are determined from the polarized phasor ellipse and $r$ can be computed from equation (26). This value of $r$ can be compared with $r_{\text{expt}}$ and the chi-squared computed. This process is repeated until the lowest chi-squared is obtained.

\[
 \text{chi-squared} = (r - r_{\text{expt}})^2/r_{\text{expt}}.
\]

3. Materials and methods

3.1. Materials

BaF/3 cells (Ludwig Institute for Cancer Research, Melbourne, Australia) were washed twice (by centrifugation) with RPMI 1640 (Invitrogen) phenol-red-free medium. Cells were stained with fluorescein diacetate (Invitrogen) by mixing a 50 $\mu$L solution of a 0.24 mM fluorescein diacetate with a 100 $\mu$L suspension of cells and allowed to incubate for 5 min. The stained cells were then loaded onto a Live Cell Microarray slide (Nunc) and washed thrice with 200 $\mu$L fresh medium prior to imaging. Lifetime, anisotropy, parallel and perpendicular polarized images of the cells were obtained using a frequency-domain wide-field microscope with corrections for background and G-factor as described previously [13].

3.2. Methods (data analysis)

The data obtained from our experiments were:

(i) Steady-state anisotropies from a sample of individual cells from parallel and perpendicular-polarized images.

(ii) Parallel and perpendicular-polarized phasors from a sample of individual cells.

The analysis proceeded in three stages.

In the first analysis the steady-state anisotropy was used in combination with the initial anisotropy and lifetime to determine the apparent isotropic rotational correlation time. Using the Perrin equation and values of $r_0 = 0.4$ and $\tau = 4$ ns for fluorescein, the apparent isotropic rotational correlation time was obtained from the cell-population-averaged anisotropy $r = 0.11$.

Substitution of values ($r_0 = 0.4$, $\tau = 4$ ns, $r = 0.11$) into equation (2) yielded, $\phi = 1.5$ ns.

In the second data analysis stage, the dynamic depolarization data, i.e. the parallel and perpendicular polarized phasors of the cells, were analysed using equations (19)–(21) to obtain apparent lifetimes and rotational correlation times. These values were then averaged over the cell population to obtain an average value for the lifetime and rotational correlation time.

Because the two estimated values for the rotational correlation time from steady-state data were not in agreement, the next level of analysis was attempted.

In the third analysis stage, the perpendicular polarized phasor ellipse for an isotropic rotator was drawn using equations (15) and (16) with fixed values of $r_0 = 0.4$, $\tau = 3.9$ ns and variable $\phi = [0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100]$ ns. As expected the cell-averaged perpendicular-polarized phasor was located inside the perpendicular polarized phasor ellipse. To locate possible values of $\phi_1$, $\phi_2$ and $\beta$ that were consistent with the experimental phasor, chords were drawn from different points on the polarized phasor ellipse corresponding to $\phi_1 = 0.1, 0.2, 0.5, 1, 2$ ns through the experimental perpendicular-polarized phasor and the corresponding value of $\phi_2$ deduced from the point of intersection of the chord with the perpendicular polarized phasor ellipse. The value of $\beta$ for each pair of correlation times was determined from the relative distance of the experimental phasor along the chord connecting phasors $\phi_1$ and $\phi_2$. This procedure generated a set of parameters that were consistent with the experimental polarized phasor. This constraint meant that each $\phi_1$ was mapped onto a unique set of $\phi_2$, $\beta$. The difference between the theoretical anisotropy and the experimental anisotropy was then used as a goodness of fit parameter to judge which model was most consistent with all of the data.

4. Results

To provide an illustrative example of the concepts discussed in the theory we turn to the measurement of the anisotropy decay of a small cytoplasmic fluorescent probe in living cells. Fluorescein derivatives have been employed by several groups to probe the microviscosity of the cytoplasm of living cells by means of steady-state anisotropy [17], multi-frequency domain phase micro-fluorimetry [18] and gated time-resolved wide-field anisotropy [19].

We employed a live-cell array in conjunction with a frequency-domain wide-field microscope fitted with an emission polarization image splitting device to obtain rotational dynamics and lifetime information from a number of individual cells. The data extracted from our measurements were (i) steady-state anisotropies of...
individual cells and (ii) polarised phasor values from a sample of cells. Figure 4 illustrates transmitted light, fluorescence and steady-state images of a live-cell array containing fluorescein diacetate-stained BaF/3 cells. From a number of cells, an average anisotropy of \( \approx 0.1 \) (mean anisotropy = 0.11, \( N = 21 \) cells) was computed (see also [17]). If we assume the simplest model for isotropic rotation (with \( r_o = 0.4, \tau = 4 \text{ ns} \) for fluorescein) we obtain an apparent single rotational correlation time of 1.5 ns (table 1, equations (1) and (2)). A second means to estimate single rotational correlation times is from the dynamic measurement of parallel and perpendicular phasors (table 1, equations (19)–(21)). We can arrive at estimates of apparent rotational correlation times (and lifetimes) with no assumptions about limiting anisotropies. Figure 5 contains a plot of the lifetime (x-axis) and apparent rotational correlation time (y-axis) for a number of individual cells analysed using the parallel and perpendicular-polarized components of the phasor. It is clear that there is a large heterogeneity in apparent rotational correlation times extending into non-physical parameter space (range: \(-3.1, 3.3 \text{ ns}\)) but the lifetime values exhibited a smaller spread (range: \(3.6\text{--}4.1 \text{ ns}\)). Taking the average lifetime and rotational correlation time data from 50 cells, we obtained an average lifetime of 3.9 ns and an average apparent rotational correlation time of 0.76 ns. It is noted that the correlation times estimated from dynamic measurements and static measurements differed by nearly a factor of 2. Therefore, it is likely that a single correlation time model is inadequate to account for the rotation of the fluorescein diacetate in the BaF/3 cells.

A third means to evaluate the isotropic rotation model graphically is to plot the polarized phasor ellipse for the family of single correlation times and examine the experimental phasor relative to this plot (table 1, equation (17) or (18)). Figure 6 illustrates the average perpendicular-polarized phasor from the fluorescein diacetate probe from a number of BaF/3 cells (30 cells). It is clear from examination of the position of the phasor that the anisotropy decay cannot be isotropic since the experimental phasor is located inside the (single correlation-time) polarized phasor ellipse (constructed using equation (18) with \( r_o = 0.40 \) and \( \tau = 3.9 \text{ ns} \)). The next logical model to consider is the single lifetime, two correlation time model. For this model we require \( r_o, \tau, \ldots \)
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one of the polarized phasors and the steady-state anisotropy $r$ (table 1).

As discussed in the theory, a two correlation-time model is represented graphically by a chord that intersects at two positions on the polarized phasor ellipse and with the experimental data point (figure 3). Examples of five such chords are provided in figure 6. If we examine one chord, where the two intersection points on the ellipse correspond to perpendicular-polarized phasors with $\phi_1 = 0.2$ ns, $\phi_2 = 50$ ns we can see that the experimental data point is located about half way between the two phasor positions on opposite sides of the ellipse. From the position of the experimental point along the chord, the fraction contribution of $\phi_1$ and $\phi_2$ to the anisotropy decay, and the corresponding steady-state anisotropy can all be computed. For example, for the $\phi_1 = 0.2$ ns, $\phi_2 = 50$ ns model, the fraction of $\phi_1$ was 0.5 and the computed steady-state anisotropy was 0.195. We can repeat this process by guessing a new value of $\phi_1$ and using the polarized phasor plot to determine the second correlation time and its fraction. Table 2 contains details of pairs of correlation times, fractional contributions and computed steady-state anisotropies that are consistent with the observed perpendicular polarized phasor. However it is clear that the theoretical steady-state anisotropy is also strongly influenced by the parameters, as expected. We can use the measured steady-state anisotropy to exclude certain pairs of correlation times and therefore narrow down to the best model. From table 2 we can see that the computed anisotropy for 4 out of the 5 correlation-time pairs were clearly inconsistent with the measured steady-state anisotropy of 0.11. Therefore using this purely graphical approach we can deduce that the most likely double-exponential rotational correlation-time anisotropy decay model involves a major contribution from a short correlation time of 0.1 ns and a minor contribution from a longer correlation time of 20 ns (Ch-squared = 0.002).

5. Discussion

The polarized phasor plot enables visualisation and analysis of fluorescence anisotropy decay at a single modulation frequency. The position of the polarized phasor with respect to polarized phasor ellipse indicates whether the anisotropy decay is simple (one correlation time, data point on the ellipse) or complex (more than one correlation time, data point inside the ellipse). A significant advantage of the polarized phasor representation is that when the steady-state anisotropy is also measured then parameters from a double-exponential correlation time model can be extracted. Importantly, the geometric constraints imposed by vector algebra and the polarized phasor ellipse reduce the determination of a double-exponential anisotropy decay from a three parameter minimisation problem to a one parameter minimisation problem. This is because there is a fixed relationship between the first correlation time, the second correlation time, the fractional contributions of each correlation time, and the steady-state anisotropy. Effectively one only needs to select the first correlation time, use the phasor plot and experimental polarized phasor to determine the second correlation time and fractional contribution, compute the theoretical anisotropy, and then alter the first correlation time and repeat minimising the difference between the theoretical steady-state anisotropy and the measured steady state anisotropy.

The anisotropy decay of fluorescein diacetate in BaF/3 cells revealed complex behaviour. This was inferred by first assuming a single correlation time model and then showing that the correlation time extracted from this model was incompatible with the steady-state anisotropy. By combining the two measurements, the polarized phasor and the steady-state anisotropy, the parameters from a double exponential correlation time model were obtained, to our knowledge, for the very first time (i.e. without recourse to multiple frequencies). The complex anisotropy decay behaviour inferred from the fluorescein diacetate in BaF/3 cells is not inconsistent with observations from other researchers. Verkman and colleagues used

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**Table 2.** Dual rotational correlation time models for fluorescein diacetate in BaF/3 cells.

| Correlation time 1 | Correlation time 2 | Fraction 1 | Fraction 2 | Anisotropy |
|--------------------|--------------------|------------|------------|------------|
| 0.1                | 20                 | 0.74       | 0.26       | 0.094      |
| 0.2                | 50                 | 0.5        | 0.5        | 0.195      |
| 0.5                | 100                | 0.26       | 0.74       | 0.296      |
| 1                  | 100                | 0.17       | 0.83       | 0.332      |
| 2                  | 100                | 0.11       | 0.89       | 0.342      |

*Note: the strong dependence of model parameters on the computed anisotropy. See text for details.*

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Figure 6. Analysis of double-exponential correlation times. Squares denote the positions of single correlation time perpendicular-polarized phasor ellipse for $r = 0.4$, $\tau = 3.9$ ns, $\omega = 40$ MHz. The circle represents the average ± two standard errors of the perpendicular-polarized phasor for fluorescein-diacetate in BaF/3 cells. Chords drawn from the edges of the ellipse through the experimental data point denote specific models of double exponential anisotropy decay. Values in italics represent the computed steady-state anisotropy for 4 out of the 5 correlation-time pairs. From table 2 we can see that the computed anisotropy for 4 out of the 5 correlation-time pairs were clearly inconsistent with the measured steady-state anisotropy to exclude certain models of double exponential anisotropy decay. Values in italics represent the computed steady-state anisotropy. See text and table 2 for more details.
single-point multi-frequency measurements with fluorescein derivatives to probe the micro-viscosity of adherent cells. They found a two correlation time model was adequate to describe the data with a major contribution (0.65–0.74 fraction) of a short correlation time in the range of 0.18–0.25 ns and a longer correlation time of 15–25 ns [16]. These results agree with our conclusion deduced from our single frequency approach that there is a major sub-nanosecond correlation time component and a longer rotational correlation time in the tens of nanoseconds with a smaller contribution to the anisotropy decay. Although comparison of different probes, different approaches and different cells should always be treated with caution, the agreement at least in the magnitude of the correlation times extracted between the two approaches lends some support to the theoretical model being tested here-in.

We envisage that the approach derived here may find particular utility in situations where greater photon efficiency, wide-field imaging and more rapid temporal acquisition is required. Acquisition times are comparable to standard frequency-domain FLIM, which at the state of the art is video rate. These circumstances may arise when rotational dynamics of macromolecules are changing in response to cell activation processes on the timescale of seconds-to-minutes. The polarized phasor ellipse plots may also find utility in examining and interpreting changes in rotational motion that accompany interactions between macromolecules in solution.

Finally we note that this method is intended to be complementary to existing approaches to examining rotational motions [19].

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