Design of a fluorescence technique using double laser pulse excitation for the measurement of molecular Brownian dynamics

J.R. Torga *, J. I. Etcheverry ** and M.C. Marconi ***

* , *** Departamento de Física - ** Departamento de Matemática

Facultad de Ciencias Exactas y Naturales- UBA

Ciudad Universitaria- Pabellón 1

1428, Buenos Aires- Argentina

Abstract

A complete analysis of a novel method to evaluate the rotational diffusion time of fluorescent molecules is presented. It is based on the study of the total fluorescence produced after excitation with two consecutive short laser pulses. Expressions for the total fluorescence energy emitted in this case are derived, that would allow the evaluation of the rotational diffusion time. The main advantages of the proposed method are its simple setup, a time resolution only limited by the exciting laser pulse-width, and the fact that it does not require fast time response detectors.

PACS: 33.15.Vb, 32.50.+d, 42.62.-b

Keywords: Rotational depolarization, Brownian dynamics, fluorescence anisotropy.

* Fellow from CONICET

*** Member of Consejo Nacional de Investigaciones Cientificas, CONICET

Corresponding author: J.R. Torga

FAX: (54) 1-782-7647

torga@df.uba.ar

The study of the fluorescence anisotropy produced in a fluorescent sample is a very helpful method to obtain information about the rotational dynamics of fluorophores. This technique for example may be used to evaluate the rotational Brownian motion of molecules with fluorescent groups rigidly attached, so obtaining information about the volume and shape of the carrier molecules, or can reveal if the fluorophore can rotate freely or if it is restricted in its angular displacement by the surrounding environment [1,2].

When a pulse of linearly polarized light impinges a sample composed of absorbing molecules, the angular anisotropy imposed by the light electric field direction produces a photo-selection in the sample inducing an anisotropy displayed by a non-isotropic polarization of the fluorescence. The degree of polarization of this signal can be measured under steady-state conditions using CW excitation light sources to obtain a mean value. However, the most important aspect of fluorescence anisotropy techniques are time-resolved studies, which allow the direct measurement of the rotational correlation times but require detectors faster than the characteristic decay times. A meaningful asset in this field was the utilization of ultra-short pulsed laser sources, that allow the investigation of processes with time resolutions in the picosecond range.

Extensive work has been done in the theoretical aspect by Perrin [3], Jablonski [4], Weber [5], Chuang et al. [6] and Tao [7], who derived expressions for the polarization of the fluorescence for different fluorophore geometries. A very

comprehensive review in this area is the paper by Kawski [8]. Time-resolved rotational diffusion was also measured using stimulated Raman scattering [9] and anisotropic picosecond absorption [10,11].

In this paper we propose a novel method, that consists in the study of the total fluorescence produced when the sample is illuminated with two short laser pulses, as a function of the delay between the pulses. This approach has the advantages of a simple experimental set up and the possibility of obtaining rotational correlation times with a time resolution only limited by the excitation pulse-width.

Two linearly polarized short laser pulses with the same polarization are successively focused in a fluorescent sample, separated in time by a delay t_0. The first pulse produces a photo-selected ensemble of excited molecules along a direction in space defined by the light polarization. The consequent anisotropy changes the characteristics of the fluorescence (polarization and intensity) originated by the second laser pulse, as this pulse will find a non homogeneously distributed ensemble of non excited molecules, provided that the delay time between pulses t_0 is smaller than the fluorescence decay and rotation times. The fluorescence produced with the double pulse excitation is time integrated with a slow-time-response detector and is analyzed as a function of the delay between pulses. This has the advantage of not requiring fast detectors.

In this work we present a complete analysis of this proposed method to show

how the rotational diffusion time can be retrieved from the total fluorescence measurements with two laser pulses excitation. In this calculation we assume spherical molecules and very short excitation pulses. We also present numerical simulations corresponding to non spherical fluorophores and non-delta excitation pulses, and a feasibility analysis for a typical case.

After the excitation by the first pulse, the initial angular distribution of excited molecules changes in time due to Brownian rotational movement, then modifying the distribution of excited molecules and relaxing again to an isotropic distribution. Following Tao [7], the angular distribution of excited molecules immediately after the excitation with the first delta pulse is given by:

$$n_1(\theta, t=0) = n_0\ [1 - \exp(K\ \cos^2(\theta))] \qquad (1)$$

where $n_1(\theta,t=0)$ represents the density of excited dipoles at an angle θ relative to the polarization axis of the exciting pulse, and n_0 is the initial isotropic population of absorbing molecules. $K=I\sigma/h\upsilon$ is a factor that accounts for the fraction of molecules excited in the sample and can be calculated from the total energy of the excitation pulse I and the absorption cross section of the fluorescent molecule σ. For example, for typical values $\sigma = 10^{-16}$ cm^2 and λ=570 nm, an intensity 3.4 10^{-4} J/cm^2 is necessary to achieve K=0.1.

We describe the population of excited fluorophores $n(\theta,\phi,t)$ in the usual way [6,8]. In the case of spherical molecules, the rotational diffusion term reduces to a diffusion constant D times the angular part of the laplacian of the

molecular density, and the whole problem has rotational symmetry in the direction of the excitation laser polarization. Accordingly, we have:

$$\frac{\partial n(\theta,t)}{\partial t} = K I(t) \cos(\theta)^2 (n_0 - n(\theta,t)) - \frac{n(\theta,t)}{\tau} + D \Delta_{\theta,\varphi} n(\theta,t) \qquad (2)$$

where τ is the characteristic fluorescence time.

As we are assuming delta pulse excitations, the evolution of the population of excited molecules away from the excitation pulses is given only by the diffusion and fluorescence decay terms. Under these assumptions, and using the linearity of the problem, it is easy to find a general expression for the density of molecules excited by the first and the second laser pulses, n_1 and n_2 respectively, in terms of the eigenfunctions $\Psi_j(\theta)$ of the rotational diffusion operator:

$$n_i(\theta,t) = n_0 \exp(\frac{t - t_i}{\tau}) K \sum_j c_j^{(i)} \Psi_j(\theta) \exp(E_j (t - t_i)) \qquad i = 1,2 \qquad (3)$$

E_j are the eigenvalues corresponding to $\Psi_j(\theta)$, $c_j^{(i)}$ are constants to be determined in order to satisfy adequate initial data, and t_i, i=1,2, represent the arrival time of each laser pulse, t_1=0 and t_2=t_0. In the particular case we are considering, the orthogonal eigenfunctions are the Legendre polynomials, $\Psi_j(\theta)$=$A_j P_j(\cos(\theta))$, where A_j is a normalization factor and the corresponding eigenvalues are E_j= j.(j+1)D.

With this formalism, we obtain the density of excited molecules due to the first laser pulse, using (1) as initial data. Developing this expression up to second

order in the excitation K we have:

$$n_1(\theta,t) = n_0 \exp(-\frac{t}{\tau})\, K\, [\frac{1}{3} - \frac{1}{10}K + (\frac{2}{3} - \frac{2}{7}K)\, P_2(\cos(\theta))\, \exp(-\frac{t}{\phi}) - \frac{4}{35}K\, P_4(\cos\theta)\exp(-\frac{10t}{3\phi})] \quad (4)$$

Here τ is again the fluorescence decay time and ϕ is the characteristic rotational diffusion time that, for a spherical rotor, is related to the diffusion coefficient D through ϕ=1/6D.

After a delay $t=t_0$, the second pulse arrives to the sample. At this time, the distribution of non excited molecules it is not longer homogeneous, provided that the time delay between pulses is smaller than the fluorescence decay time and the rotational diffusion time. The angular distribution of excited dipoles at the arrival of the second pulse is given by $n_1(\theta,t_0)$. The density of absorbing molecules at this time will consequently be given by $n_a(\theta, t_0) = n_o - n_1(\theta, t_0)$. Following the derivation of equation (1), the density of the molecules excited by the second laser pulse is obtained as:

$$n_2(\theta,t_0) = (n_0 - n_1(\theta,t_0))\, [1 - \exp(K\, \cos^2(\theta))] \quad (5)$$

The expression for $n_2(\theta,t)$ for $t > t_o$, obtained according to equation (3) with initial condition given by (5), and considering only up to second order in K, is:

$$n_2(\theta,t) = C_0 + C_2 P_2(\cos(\theta))\exp(-\frac{(t-t_0)}{\phi}) + C_4 P_4(\cos(\theta))\exp(-\frac{10}{3}\frac{(t-t_0)}{\phi}) \quad (6)$$

where the coefficients C_j are given by:

$$C_0 = n_0 K \left[\frac{1}{3} - \frac{1}{10} K - \frac{4}{45} K \exp\left(-t_0\left(\frac{1}{\tau} + \frac{1}{\phi}\right)\right)\right] \quad (7)$$

$$C_2 = n_0 K \left[\frac{2}{3} - \frac{2}{7} K - \frac{2}{9} K \exp\left(-\frac{t_0}{\tau}\right) - \frac{22}{63} K \exp\left(-t_0\left(\frac{1}{\tau} + \frac{1}{\phi}\right)\right)\right] \quad (8)$$

$$C_4 = n_0 K \left[-\frac{4}{35} K - \frac{8}{35} K \exp\left(-t_0\left(\frac{1}{\tau} + \frac{1}{\phi}\right)\right)\right] \quad (9)$$

The total fluorescence intensity produced by each pulse, with polarizations in the direction perpendicular ($I_{y,i}(t)$) and parallel ($I_{z,i}(t)$) relative to the polarization of the exciting pulse are given by:

$$I_{z,i}(t) = h\nu \int \frac{n_i(\theta, t)}{\tau} \cos(\theta)^2 \, d\Omega \quad (10)$$

$$I_{y,i}(t) = h\nu \int \frac{n_i(\theta, t)}{\tau} \sin(\theta)^2 \sin(\varphi)^2 \, d\Omega \quad (11)$$

After integration in time, the contribution of the i-th pulse to the total fluorescence energy emitted in both polarizations is finally obtained:

$$E_{z,i}(\tau, \phi, t_0) = h\nu \int_{ti}^{\infty} \int_{\Omega} \frac{n_i(\theta, t)}{\tau} \cos(\theta)^2 \, d\Omega \, dt \quad (12)$$

$$E_{y,i}(\tau, \phi, t_0) = h\nu \int_{ti}^{\infty} \int_{\Omega} \frac{n_i(\theta, t)}{\tau} \sin(\theta)^2 \sin(\varphi)^2 \, d\Omega \, dt \quad (13)$$

where we have explicitly shown the dependence of the total fluorescence energy on the fluorescence time, on the diffusion time, and on the time delay between pulses. The total fluorescence energy with each polarization is then obtained by summing the contributions of each pulse:

$$E_z(\tau, \phi, t_0) = \frac{h\nu}{\tau} \int_{\Omega} \left(\int_0^{\infty} n_1(\theta, t) \, dt + \int_{t0}^{\infty} n_2(\theta, t) \, dt \right) \cos(\theta)^2 \, d\Omega \quad (14)$$

$$E_y(\tau,\phi,t_0) = \frac{h\nu}{\tau}\int_\Omega \left(\int_0^\infty n_1(\theta,t)\ dt + \int_{t0}^\infty n_2(\theta,t)\,dt\right)\sin(\theta)^2\sin(\varphi)^2\,d\Omega \qquad (15)$$

The method we are discussing in this paper consists of the measurement of the total fluorescence when two pulses excite the sample, as a function of the time delay t_0. The total fluorescence produced under these conditions is compared with the fluorescence that would be produced in the case of two very delayed pulses. The difference is due to the fact that the second pulse arrives to the sample when the anisotropy in the angular distribution of non excited molecules induced by the first pulse is still present. Accordingly, we can define a parameter of interest, the differential fluorescence signal Σ for both polarizations, as:

$$\Sigma_k(\tau,\phi,t_0) = -\frac{E_k(\tau,\phi,t_0) - E_k(\tau,\phi,+\infty)}{E_k(\tau,\phi,+\infty)} \qquad k = y,z \qquad (16)$$

The explicit expressions obtained from (14) and (15) for the spherical molecules case are:

$$\Sigma_z(\tau,\phi,t_0) = K\ \exp(-\frac{t_0}{\tau})\ \frac{\tau}{\tau+\phi}\ \frac{\frac{\phi}{5}+\frac{\tau}{9}+\frac{8}{35}\exp(-\frac{t_0}{\phi})\ \phi+\frac{4}{45}\ \tau\ \exp(-\frac{t_0}{\phi})}{(\frac{1}{3}-\frac{K}{10})\ \tau\ +(\frac{4}{15}-\frac{4}{35}\ K)\frac{\tau\phi}{\tau+\phi}} \qquad (17)$$

$$\Sigma_y(\tau,\phi,t_0) = K\ \exp(-\frac{t_0}{\tau})\frac{\tau}{\phi+\tau}\ \frac{\frac{\phi}{15}+\frac{\tau}{9}+\frac{2}{105}\exp(-\frac{t_0}{\phi})\ \phi+\frac{4}{45}\tau\ \exp(-\frac{t_0}{\phi})}{(\frac{1}{3}-\frac{K}{10})\ \tau-(\frac{2}{15}-\frac{2}{35}K)\ \frac{\tau\phi}{\tau+\phi}} \qquad (18)$$

This differential signal depends on the fraction of excited molecules, the time

delay t_0, and the decay times τ and ϕ. In consequence, knowing the fluorescence decay time and calculating the fraction of excited molecules in the sample, it is possible to evaluate the rotation time ϕ from the measurement of the dependence of Σ on the time delay t_0.

As a typical example, let us consider rhodamine 6G in ethylene glycol at 290 K, with fluorescence and rotational diffusion times both about 4 ns, [8]. We consider an excitation pulse of 10 nJ, centered at λ=570 nm, focused to 100 μm diameter in a sample 1 mm long with a 10^{-5} molar concentration. Assuming an absorption cross section $\sigma = 10^{-16}$ cm^2, we obtain K=0.014, which corresponds to a fraction of excited molecules of 0.5 %. According to (17), (18), and assuming a collecting efficiency of 10 %, the differential energy would be 10^{-14} J, with a total fluorescence energy of 10^{-12} J. This difference can be detected averaging the signal using a high repetition rate laser excitation.

Figure 1 shows plots of expressions (17) and (18) as a function of the time delay t_0 normalized to the fluorescence decay time τ, for different ratios ϕ / τ. For fluorescence times small compared with ϕ, the molecule will emit before a significant rotation takes place. In these conditions the differential fluorescence signal is large, and the effect of the double excitation is important for time delays up to values of order τ. If the fluorescence time is large compared with the rotation time, the dipole will significantly rotate before de-excitation by fluorescence. Under these conditions the differential signal is only noticeable

for short delays up to values of the order of ϕ.

Notice that for different ratios ϕ/τ, the differential fluorescence curves are more sensitive as ϕ/τ decreases. For example, changing the value of ϕ/τ from 5 to 10, produces a maximum change in the differential fluorescence signal of 6 %, while Σ changes about 18% when ϕ/τ varies from 1/5 to 1/10.

This can be easily seen in Figure 2 where the sensitivity $S= \partial\Sigma/\partial\phi$ is plotted as a function of t_0/τ for different values of ϕ/τ. The figure shows that for delay times longer than τ, the technique will not be useful because all curves go to zero. This is expected from the fact that information is retrieved from the fluorescence signal, that goes to zero for $t_0 >> \tau$.

In order to evaluate the range of ϕ/τ values where this method will be useful, we plot in Figure 3 the normalized sensitivity S/Σ, as a function of t_0/τ for different ratios ϕ/τ. The curves show that maximum sensitivity is obtained for delay times t_0 of the order of ϕ. All the plots shown correspond to a fraction of excited molecules of 3%.

From the two previous figures, we conclude that the three times involved t_0, τ, ϕ, should be about the same order to obtain good signal and sensitivity.

The same behavior for the differential energy Σ is obtained if we consider a finite pulse width excitation. Figure 4 shows the numerical results for gaussian excitation pulses having $\tau/2$, $\tau/4$, and $\tau/16$ FWHM. The convergence of the numerical results for decreasing pulse width to the delta excitation results is

clearly observed. Particularly, for $t_0 \leq \tau/20$ the differences between the two results are negligible.

In conclusion, we have presented a complete analysis of a proposed alternative method that allows the evaluation of the ratio between the rotational diffusion time and the fluorescence decay time for fluorescent molecules, using double laser pulse excitation. As a representative example, the calculations for the simple case of an spherical rotor are presented, but this formalism can be applied to other fluorophore geometries. In particular, the case of symmetric top molecules with the excitation dipole along the main symmetry axis has the same formal expressions, where the diffusion constant D is the corresponding element of the diffusion tensor along the symmetry axis. Numerical calculations for finite pulse width excitation are also presented, showing that for realistic pulse widths the expected signal is very close to the analytical results for delta pulse excitation.

An interesting feature of the proposed method is that it does not require a fast detection system, and the information is retrieved from the time integrated fluorescence signal. Resolution is only limited by the pulse width of the laser excitation, although better signal and sensitivity are obtained when t_0, τ, ϕ are all about the same order.

Acknowledgments

This work was supported by Universidad de Buenos Aires (PMA Program and grant EX285) and a grant from Fundación Antorchas.

We thank P.F. Aramendia and M. Negri for helpful discussions on several aspects of this work.

FIGURE CAPTIONS

Figure 1- Differential fluorescence signal a) parallel (Σ_z) and b) perpendicular (Σ_y) to the excitation direction as a function of the delay time between pulses for different values of the rotation time. a: ϕ/τ = 10, b: ϕ/τ = 5, c: ϕ/τ =1, d: ϕ/τ =1/ 5, e: ϕ/τ =1/ 10. Times are normalized to the fluorescence decay time. K=0.1.

Figure 2- Sensitivity $S= \partial\Sigma/\partial\phi$, a) parallel ($S_z$) and b) perpendicular ($S_y$) to the excitation direction, as a function of the time delay between pulses, for different values of the rotational diffusion time: a - ϕ/τ = 2, b - ϕ/τ =1, c - ϕ/τ = 1/5, d: ϕ/τ = 1/10. Times are normalized to the fluorescence decay time. K=0.1.

Figure 3- Normalized sensitivity S/Σ, a) parallel (S_z/Σ) and b) perpendicular (S_y/Σ) to the excitation direction, as a function the time delay between pulses, for different values of the rotational diffusion time. a: ϕ/τ = 2, b: ϕ/τ =1, c: ϕ/τ = 1/5, d: ϕ/τ = 1/10. K=0.1.

Figure 4- Differential fluorescence signal parallel (Σ_z) to the excitation direction as a function of the delay time between pulses for gaussian excitation pulses of widths (FWHM): $\tau/2$ (circles), $\tau/4$ (crosses), $\tau/16$ (squares). Times are normalized to the fluorescence decay time, and $\phi=\tau$. Solid line: delta excitation.

REFERENCES

1- Principles of Fluorescence Spectroscopy. J.R. Lakowicz. Plenum Press, New York London, 1986

2- Perrin, F. Ann. Phys. 1929,12,169

3- Perrin, F. Acta Phys. Pol.1936, 5, 335

4- A. Jablonski, 1960, Bull. Acad. Pol. Sci. 8, 259

5- G. Weber, J. Phys. Chem 93, 6069-6073, 1989.

6- T.J. Chuang and B.K. Eisenthal, J. Chem Phys. 57, 5094-5097, 1972

7- T. Tao, Biopolymers 8, 609-632, 1969.

8- A. Kawski, Crit. Rev. in Anal. Chem., 23, 459-529, 1993.

9- E.A.J. Spaans, M.P. van Exter and A. Lagendijk, Optics Comm. 76, 376-380, 1990.

10- C.V. Shank and E.P. Ippen. Appl. Phys. Lett 26, 62, 1975.

11- D.H. Waldeck and G.R. Fleming. J. Phys. Chem. 85, 2614-2617, 1981.