Journal of Fluorescence, Vol. 4, No. 3, 1994

Triplet-State Monitoring by Fluorescence Correlation Spectroscopy[1]

Jerker Widengren,[2] Rudolf Rigler,[2,3] and Ülo Mets[2]

Received October 18, 1993; revised June 1, 1994; accepted June 1, 1994

The effects of high excitation intensities in fluorescence correlation spectroscopy (FCS) in terms of saturation and triplet-state build-up have been studied for the case of Rh6G in aqueous solution. It was found that FCS provides a powerful means for the determination of intersystem crossing and triplet-state depopulation rates of fluorophores in solution.

KEY WORDS: Fluorescence correlation spectroscopy; triplet state.

INTRODUCTION

In fluorescence correlation spectroscopy (FCS) intensity fluctuations of fluorescent molecules excited by a laser beam are studied [1,2]. In principle, information can be obtained about any dynamic process, lying in the submicrosecond time range and above, that gives rise to fluctuations in fluorescence intensity. In particular, transport processes (translational and rotational motion), chemical reactions, and binding interactions can be monitored, but also other parameters can be measured [3]. The introduction of extremely small-volume elements [4] has increased the sensitivity of FCS by several orders of magnitude, allowing the detection and analysis of single molecules [3,5]. To obtain optimum conditions during measurements, it is necessary to apply excitation intensities upon the fluorescent molecules, leading to photophysical processes, the effects of which must be taken into consideration. In this study it is shown that the main distortions seen on the correlation curves can be attributed to a buildup of the populations in the triplet states of the fluorescent molecules under investigation. Furthermore, it is shown that it is possible to measure the rates of intersystem crossing, triplet-state depopulation, and either the excitation cross section or the lifetime of the singlet state by FCS.

EXPERIMENTAL

The fluorescence of rhodamine 6G (Rh6G) molecules in distilled water was studied in air atmosphere. The experimental setup is shown in Fig. 1. The laser beam of an argon ion laser emitting at 514 nm is focused by an epiilluminated microscope into the sample containing the molecules under study. The dimensions of the laser beam focus and the pinhole diameter in a confocal setup define the sample volume from which fluorescent light is collected (Fig. 2). The light is detected by two-photon counting avalanche diodes (EG&G SPCM-100) in a beam-splitting arrangement. To eliminate correlations due to the dead time of the diodes (250 ns), the signals of the two detectors have been cross correlated (ALV Model 5000 correlator). Cutoff filters (Schott KV550) in front of both diodes were used.

[1] This is a peer-reviewed conference proceeding article from the Third Conference on Methods and Applications of Fluorescence Spectroscopy, Prague, Czech Republic, October 18–21, 1993.

[2] Department of Medical Biochemistry and Biophysics, Karolinska Institute, S–171 77 Stockholm, Sweden.

[3] To whom correspondence should be addressed.

1053-0509/94/0900-0255$07.00/0

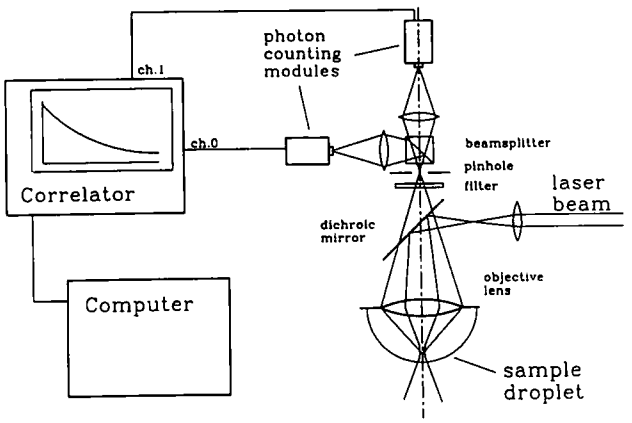

Fig. 1. Experimental setup for FCS.

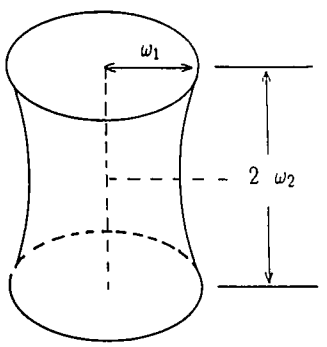

Fig. 2. Volume element of the laser beam.

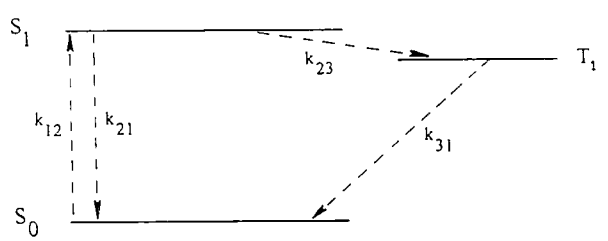

Fig. 3. The electronic states of Rh6G and its transition rate constants.

THEORETICAL BACKGROUND

The electronic states of Rh6G involved in the processes of fluorescence can be modeled as shown in Fig. 3. S_0 denotes the ground singlet state, S_1 is the excited singlet state, and T is the triplet state. k_{12}, k_{21}, k_{23}, and k_{31} are the rate constants for excitation and deexcitation of the singlet state, intersystem crossing, and deexcitation of the triplet state, respectively.

The population of the three states can be expressed in terms of the rate constants by solving the following system of three first-order differential equations:

$$\frac{d}{dt}\begin{pmatrix} S_0(t) \\ S_1(t) \\ T(t) \end{pmatrix} \begin{bmatrix} -k_{12} & k_{21} & k_{31} \\ k_{12} & -(k_{23}+k_{21}) & 0 \\ 0 & k_{23} & -k_{31} \end{bmatrix} \begin{pmatrix} S_0(t) \\ S_1(t) \\ T(t) \end{pmatrix} \quad (1)$$

Assuming a rhodamine molecule in the laser beam being subject to a constant intensity starting at time $t=0$, it follows from the solution of the equations above that the likelihood of having any of the states populated at time $t=t_1$ can be expressed as

$$X(t_1) = \sum_{i=1}^{3} A_i e^{\lambda_i t_1} \quad (2)$$

where A_i and λ_i are the components of the eigenvectors and the eigenvalues of the equation matrix above, respectively. The first eigenvalue, λ_1, will be zero, indicating that the populations in the states will approach a steady state as $t \rightarrow \infty$. The second eigenvalue, λ_2, will be of a high magnitude and represents the so-called "antibunching" term. In this treatment it is neglected since its magnitude will be too high to be measured properly with the time resolution of our correlator. It will be of the same order of magnitude as the sum of the inverse singlet lifetime and the excitation rate. The magnitude of the third eigenvalue, λ_3, is roughly related to the rate at which there is a population buildup in the triplet state. Assuming that $k_{12} + k_{21} >> k_{31} + k_{23}$, λ_3 can be expressed as

$$\lambda_3 = -k_{31} - \frac{k_{12}k_{23}}{k_{12} + k_{21}} \quad (3)$$

The rate of excitation, k_{12}, can be considered to be proportional to the intensity, so that λ_3 as well as T_{eq} will show an intensity dependence. When $t \rightarrow \infty$ a steady state is reached. Normalizing the populations so that $T+S_0+S_1=1$, the population in the triplet state at steady-state equilibrium can be expressed as

$$T_{eq} = \frac{k_{23}k_{12}}{k_{12}(k_{23}+k_{31}) + k_{31}(k_{21}+k_{23})} \quad (4)$$

Applying this electron state model to the case in FCS, the fluctuations in intensity will either be caused by diffusion of the fluorophores in and out of the laser beam volume or arise as the molecules are entering and leaving the triplet state. The corresponding autocorrelation function for the fluorescence intensity from the molecules can be written as

$$g(\tau) = 1 + \frac{1}{N}\left(\frac{1}{1+4D\tau/\omega_1^2}\right)\left(\frac{1}{1+4D\tau/\omega_2^2}\right)^{1/2} \times (1 - T_{eq} + T_{eq}e^{-\lambda_3\tau}) \quad (5)$$

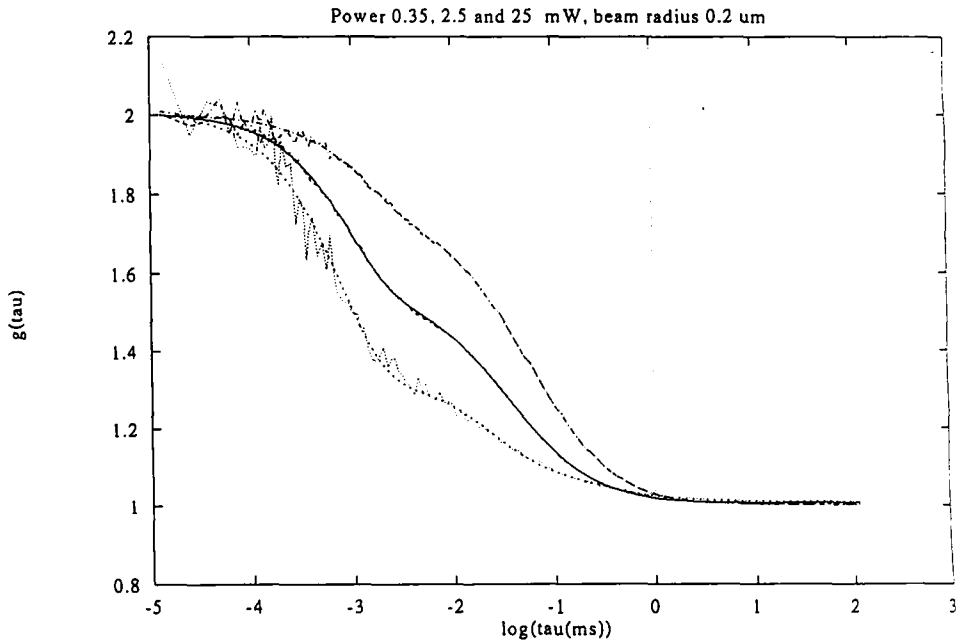

Fig. 4. Experimental and fitted correlation curves for Rh6G at different intensities. The three curves were measured at 0.35, 2.5, and 25 mW excitation power, respectively, having a beam radius of 0.24 μm. The amplitudes of the correlation functions (1/*N*) have all been normalized to 1.

Table I. Obtained Values of the Rate Constants for Rh6G[a]

Excitation cross section (10^{-16} cm^2) (at 514 nm excitation wavelength)	1.3 ± 0.4 (1.7)
k_{21} (10^6 s^{-1}) (measured by pulsed laser and TCSPC)	250 ± 20 (200)
k_{23} (10^6 s^{-1})	0.9 ± 0.2 (0.4)
k_{31} (10^6 s^{-1})	0.5 ± 0.1 (0.3)

[a] Referenced values within parentheses.

Here D is the diffusion constant of the fluorescent molecules, ω_1 and ω_2 are the distances from the center of the volume element in the radial and axial direction, respectively, at which the laser intensity has dropped by a factor of e^2, assuming a Gaussian beam profile, and N is the mean number of fluorescent molecules being in either of the singlet states inside the volume element.

RESULTS

The intensity fluctuations of Rh6G in water solutions were measured by FCS at different laser intensities. The correlation curves were fitted to Eq. (5) by a nonlinear least-squares parametrization procedure (Levenberg–Marquardt) (Fig. 4). In the fitting procedure the following parameters were allowed to vary freely: T_{eq}, λ_3, N, DC, τ_D, and ω_2/ω_1. τ_D is equal to $\omega_1^2/4D$ and DC will be $g(\tau)$ as $\tau \rightarrow \infty$. ω_2 was coupled to ω_1 as a ratio parameter, ω_2/ω_1. For an aqueous solution of Rh6G with $D = 2.8\times10^{-10}$ m^2/s, ω_1 and ω_2 could be obtained from the fitted values of τ_D (50 μs) and ω_2/ω_1 (5) to be 0.24 and 1.2 μm, respectively. This gives a sample volume element of 0.2 fl. From the fitted values of N (for most curves somewhat less than one), one could calculate the concentration to be approximately 5 nM. This was in agreement with the concentration prepared within pipetting errors. (At higher intensities the correlation function amplitude was reduced, probably due to saturation broadening of the fluorescence profile as well as to photodecomposition effects.)

To estimate the rate parameters the fitted values of the parameters λ_3 and T_{eq} were plotted as a function of the applied laser power (Fig. 5). The obtained curves were subject to a new curve fitting combining Eq. (3) and Eq. (4). In the fitting procedure k_{12} was supposed to be linear to the power of the laser, i.e., $k_{12}=K\times P$, where K, together with k_{23} and k_{31}, was allowed to vary freely and P denotes the applied laser power. The fitted value of K was translated into the excitation cross section, σ_{exc}, given by $k_{12} = \sigma_{exc} \times \Phi$, where the excitation intensity was approximated as $\Phi=P/\pi\omega_1^2$ and expressed as photons per square centimeter per second. In the fitting procedure the sum of the parameters ($k_{21}+k_{23}$) was fixed to

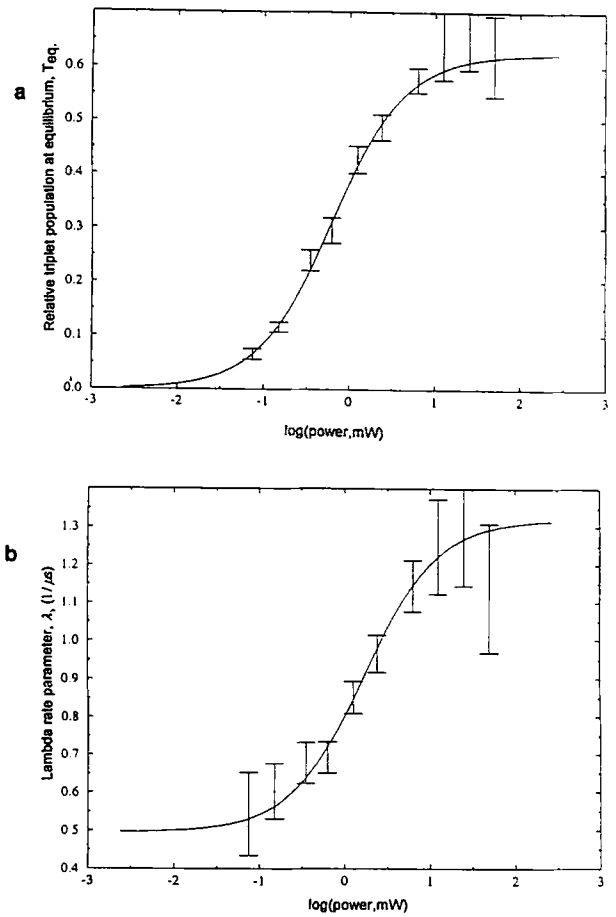

Fig. 5. Triplet-state population, T_{eq}, and relaxation rate, λ_3, as a function of excitation power. Experimental and fitted values for Rh6G. Beam radius, 0.24 μm.

a value of 2.5×10^8 s^{-1}, given by time-correlated single-photon counting measurements.

The rate parameters obtained are given in Table I. The values in parentheses are from Ref. 6 (k_{21}, k_{23}, and k_{31}) and Ref. 7 (σ_{exc}). In Ref. 6 flash photolysis was used and the measurements were made on deaerated aqueous solutions. To the reported value of k_{31} has been added the reported contribution to the triplet deexcitation rate of oxygen quenching at air equilibrium. In view of the different experimental techniques and different experimental conditions, our experimental values agree fairly well with those reported in Refs. 6 and 7.

CONCLUSIONS

The distortion of FCS curves at high laser intensities can be attributed to a buildup of the population in the triplet state. This can be accounted for by the application of a three-state electron model. Intersystem crossing rates, triplet decay rates, and excitation cross sections at concentrations close to the single-molecule level can be measured for fluorescent molecules in a solution. Knowing the rate constants is important to find the experimental conditions leading to optimum fluorescence. This is of importance in single-molecule detection experiments. Due to the environmental sensitivity (quencher concentrations, pH, etc.) of the electronic transition rate constants, measuring them by FCS might be a handy way of obtaining microenvironmental information. A deeper study of triplet-state monitoring at different environmental conditions will follow.

ACKNOWLEDGMENTS

This study was supported by funds from the Technical Research Council, the J. L. Grönberg Foundation, and the Karolinska Institute. We also would like to thank Professor N. Åslund for the loan of an avalanche photodiode.

REFERENCES

1. E. L. Elson and D. Madge (1974) *Biopolymers* **13**, 1–27.
2. M. Ehrenberg and R. Rigler (1974) *Chem. Phys.* **4**, 390–401.
3. R. Rigler, J. Widengren, and Ü. Mets (1992) in O. S. Wolfbeis (Ed.), *Fluorescence Spectroscopy*, Springer-Verlag, Berlin/Heidelberg/New York, pp. 13–24.
4. R. Rigler, Ü Mets, J. Widengren, and P. Kask (1993) *Eur. Biophys. J.* **22**, 169–175.
5. R. Rigler and Ü. Mets (1992) in *Laser Spectroscopy of Biomolecules, SPIE Vol. 1921*, pp. 239–248.
6. V. E. Korobov and A. K. Chibisov (1978) *J. Photochem.* **9**, 411–424.
7. R. P. Haugland (1992) *Handbook of Fluorescent Probes and Research Chemicals*, Molecular Probes, Inc., Eugene, Oregon, p. 218.