Eur Biophys J (1985) 12:163–166

European Biophysics Journal

Fluorescence correlation spectroscopy in the nanosecond time range: Photon antibunching in dye fluorescence

P. Kask*, P. Piksarv, and Ü. Mets

Institute of Chemical Physics and Biophysics, Estonian Academy of Sciences, Lenini puiestee 10, Tallinn 200001, USSR

Received June 20, 1984/Accepted February 26, 1985

Abstract. Possibilities for the use of fluorescence correlation spectroscopy in the nanosecond time range are demonstrated. The experiment is based on a cw argon ion laser, a microfluorimeter, two photon detectors, and a time-to-analog converting system. Experiments using solutions of rhodamine 6G and pyronine G in water at concentrations of about 20 molecules per sample volume are reported. The photon anticorrelation component decaying with a time constant close to the excited state lifetime was observed.

Key words: Fluorescence correlation spectroscopy, photon antibunching in dye fluorescence

1. Introduction

Fluorescence correlation spectroscopy (Elson and Magde 1974) has been accepted as a promising new tool for studying the dynamics of molecular processes. However, use of the method has been greatly restricted by experimental difficulties (Magde et al. 1974); photochemical instability of the fluorescent materials limits the acceptable exciting intensity and, hence, the attainable signal-to-noise ratio (Koppel 1974) for a given dye. In practice, only photostable dyes can be used, such as rhodamines, pyronines and oxazines (Kändler et al. 1982), including a few fluorescent labels, such as isothiocyanates of rhodamines (Rigler et al. 1979). Fluorescence correlation spectroscopy has been used extensively in the study of the translational diffusion of dyes or labelled macromolecules in solutions or in membranes (Magde et al. 1974; Koppel et al. 1976; Rigler et al. 1979). Although potentially promising the method has found little application in studies of rotational diffusion of macromolecules (Ehrenberg and Rigler 1974) or on intramolecular changes (Steinberg and Haas 1982).

The usual time range of fluorescence correlation spectroscopy lies between a tenth and hundreds of milliseconds; a few experiments have also been carried out in the microsecond time range (Kändler et al. 1982). This can be only partially explained by instrumental difficulties; the major problem is that the signal-to-noise ratio is proportional to the square root of the time window of the correlator (Koppel 1974). Nevertheless, the aim of the present work is to investigate the possibilities for using fluorescence correlation spectroscopy in the nanosecond time range. This time range is of great practical importance, for example, in studies of the rotational diffusion of macromolecules.

The photon anticorrelation component which is the subject of the present work, was first described theoretically about ten years ago (Ehrenberg and Rigler 1974). For example, in a case of practical importance

$$4D/w^2 \ll D_j \ll 1/\tau\,, \quad j = 1, 2, 3, \tag{1}$$

(where D is the translational diffusion coefficient of the labelled molecule; w is the radius of the exciting gaussian light beam; D_j are the rotational diffusion coefficients and τ is the fluorescence lifetime) the approximate expression for the fluorescence intensity autocorrelation function $g(t)$ takes the form

$$g(t) = \langle i \rangle^2 \left\{ 1 + (\pi L w^2 n)^{-1} \left[-\frac{9}{5} \exp(-t/\tau) + \sum_{j=1}^{5} a_j \exp(-E_j t) + (1 + 4Dt/w^2)^{-1} \right] \right\} + \langle i \rangle \delta(t)\,, \tag{2}$$

where i is the fluorescence intensity; L is the sample length; n is the concentration of the labelled molecules; E_j and a_j are eigenvalues and expansion coefficients of the asymmetric rotor. For further

* To whom offprint requests should be addressed.

details see Ehrenberg and Rigler (1974). The time-dependent part of $g(t)$ is described by the second, third and fourth terms of Eq. (2), which are called the photon antibunching term, the rotational diffusion term and the translational diffusion term, respectively. Their relative amplitude is proportional to the reciprocal of the mean number of labelled molecules per sample volume.

In general, the phenomenon of photon antibunching indicates that for a certain kind of light field, having no classical analogue, the joint probability density of photodetection $P_2(0,t)$ may increase with time, t, from $t=0$. The phenomenon has been experimentally observed in the resonance fluorescence of a sodium atomic beam (Kimble et al. 1977), providing direct evidence for the quantum nature of light. In the particular case when the light source consists of a small number of atoms or molecules, the photon antibunching term describes the evolution of a molecule after emitting a photon; the molecule is initially in the ground state and, hence, unable to emit the second photon. Despite extensive theoretical effort (for a review see Kozierowski 1981), no other light sources exhibiting photon antibunching have been reported.

2. Experimental

The optical part of our experiment (Fig. 1) uses a microfluorimeter which was primarily designed for fluorescence correlation spectroscopy in the milli- and microsecond time range (Kask et al. 1983). The beam from a cw argon ion laser at 514.5 nm is focused into a microcuvette using an optical system consisting of a long focal length lens and a microscope objective with an aperture number of 0.11. The fluorescence emission is observed using a large-aperture oil immersion microscope objective. The output light is spectrally filtered by an OS-12 orange glass filter to cut off the scattered light at 514.5 nm, and by a ZS-7 green glass filter to remove the most intense lines of Raman scattering from water (at >630 nm for 514.5 nm excitation). Using an OS-12 filter and a S-20 type photocathode, pure water shows emission intensity equivalent to rhodamine 6 G at a concentration of 6×10^{-11} M, or at a concentration of 2×10^{-11} M if the ZS-7 filter is used.

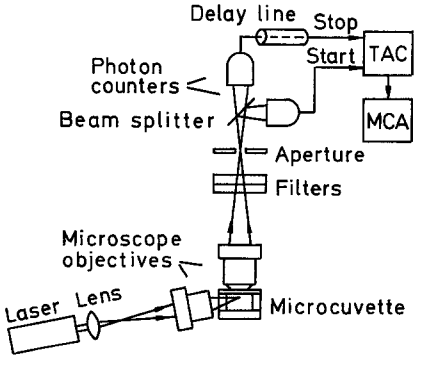

Fig. 1. The principal elements of the experiment. *TAC* – time to amplitude converter; *MCA* – multichannel analyser

An aperture is positioned in the image plane of the observation objective to separate a short segment of the image of the fluorescent trace of the laser beam for further use. The diameter of the aperture is slightly larger than aberrations during observation. The abberations are relatively large in our experiment, both because of the spatial dimensions of the exciting laser beam and because the sample is placed unusually deep in the solution, at a distance of about 0.02 mm from the cover glass, in order to avoid unwanted optical and chemical effects of the surface. With low exciting light intensities, the effective sample volume is about 200 μm^3, determined by the 3 μm diameter of the laser beam and by the 25 μm length of the sample segment. At a concentration of 2×10^{-11} M there are, on average, 2.4 molecules in this volume. The effective sample volume grows with the exciting light intensity because of the uneven concentration of unbleached dye molecules inside the exciting beam.

The light emerging from the aperture is divided into two approximately equal parts by a beam-splitter for observation by the two photon detectors.

The electronic part of the apparatus is similar to that of the first experiment on photon antibunching (Kimble et al. 1977), consisting of two photon counters, a time-to-amplitude converter (type 1701, Polon) and a multichannel pulse amplitude analyser (type NTA 1024, EMG). The photon counters consist of an FEU-79 photomultiplier with a linearly focused dynode system, a preamplifier and a constant fraction discriminator. The latter is similar to that described by Leskovar et al. (1976).

Through measurements of the light from an incandescent lamp, the most important instrumental distortion sources were found and eliminated. Using 514.5 nm light pulses from a mode-locked, cavity-dumped argon ion laser (Spectra-Physics 165-08), the time scale of the measurements and the response function of the system (FWHM 1.2 ns) were determined.

The experiments were carried out on three different substances. The first was bidistilled water, where the observed emission was caused by Raman scattering; this experiment was designed to produce a reference signal for the other experiments. For the main experiments, two relatively photostable dyes were chosen, rhodamine 6G (Sojuzreaktiv) and

pyronine G (Fluka). The choice of the dyes and conditions of the experiments were determined by fluorescence correlation experiments in the millisecond time range, where the good signal-to-noise ratio allows exact measurements of short duration (Kask et al. 1983). In this time range the fluorescence intensity autocorrelation function is a decreasing function of the delay time, as is usual in describing the dynamics of translational diffusion and photobleaching processes.

The power of the exciting cw laser beam was chosen to be 6 mW for rhodamine 6G and 12 mW for pyronine G, in order to be close to the optimum signal-to-noise ratio. At higher exciting light intensities an unfavourable increase in the effective sample volume was produced by photobleaching. No flow of solution through the cuvette was used in the experiments reported here.

The concentrations of the dyes were chosen to produce emission about five times more intense than the emission from pure water and were about 10^{-10} M inside the beam, and about 6 times higher far outside the sample volume. At lower concentrations the photon shot noise increases because of the Raman emission of water. At higher concentrations the decreasing signal amplitude increases the importance of instrumental noise.

For rhodamine the average pulse counting rate was $0.7 \times 10^5\ s^{-1}$ in the start-channel and $0.9 \times 10^5\ s^{-1}$ in the stop-channel; for pyronine the counting rates were $1.1 \times 10^5\ s^{-1}$ and $1.4 \times 10^5\ s^{-1}$. In the experiment on pure water the ZS-7 optical filter was removed and the laser beam power was 10 mW; the counting rates were $1.0 \times 10^5\ s^{-1}$ and $2.1 \times 10^5\ s^{-1}$. Here the change of asymmetry in the counting rates was caused by the spectral dependence of the transmission-reflection ratio of the beam-splitter. The duration of every experiment was 9 h, the temperature of the sample was maintained at 25 °C.

3. Results

In the case of dye fluorescence the measured function has a reproducible minimum around the zero-point on the delay time scale. As expected, this minimum is absent in experiments on pure water. The results can be described in a simple way by the expression

$$\hat{g}(t) = \langle i_1 \rangle \langle i_2 \rangle \{1 + \langle N \rangle^{-1} [1 - \exp(-|t|/\tau)]\} \cdot \exp[(\Delta - t)\langle i_2 \rangle], \quad (3)$$

where $\langle N \rangle$ is the apparent average number of the dye molecules per sample volume; i_1 and i_2 are the count rates in start- and stop-channels, respectively; Δ is the time delay in the stop-channel. Several terms from Eq. (2) have been dropped here. The last term is absent because only cross-correlations between two photon detectors are counted. The rotational diffusion term is neglected because $D_j \gg 1/\tau$. Translational diffusion is very slow compared with the time scale of the experiment, and therefore the translational diffusion term is described as constant. The last factor of Eq. (3) serves as a simplified description of the dead-time distortions of the equipment.

In addition to the photon shot noise, the results of all the measurements contained a noticeable amount, about 1%, of instrumental noise, appearing reproducibly as positive or negative deviations in the count rates of certain of the memory channels of the pulse height analyser. To diminish the role of the instrumental noise, the direct results of the experiments on the dye solutions have been divided, channel by channel, by the direct result of the experiment on pure water. The results after this procedure are represented in Fig. 2. The small positive slope of these functions is produced by the relatively high counting rate used in the stop-channel for the reference experiment on pure water. Curve fitting according to Eq. (3), with the estimated fluorescence decay times of 7.5 ± 1.5 ns for rhodamine 6G and 4.5 ± 1.0 ns for pyronine G, are also shown in Fig. 2. The non-zero response time of the apparatus was not taken into account.

The estimated apparent average number of molecules per sample volume was 21 for rhodamine 6G and 37 for pyronine G solutions in our experi-

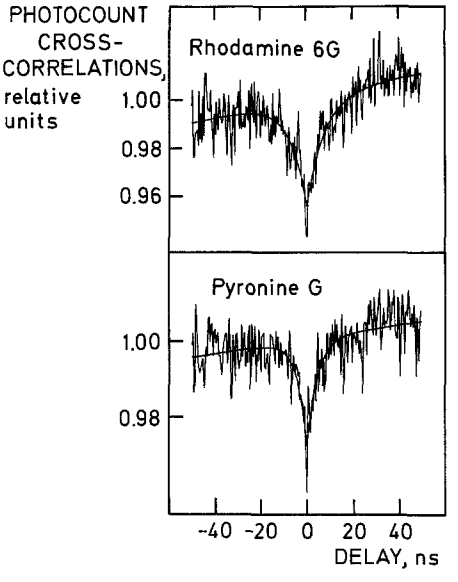

Fig. 2. The measured photocount cross-correlation function for the rhodamine 6G and pyronine G solutions. The smooth fit curves are also presented

ments. At a concentration of 10^{-10} M there are 12 molecules per 200 μm^3. The apparent values are somewhat higher because of the high constant background from water and because of distortions caused by photobleaching.

We have also estimated the fluorescence decay time of the dyes from an ordinary relaxation experiment (Leskovar et al. 1976) using pulse excitation with the mode-locked argon ion laser. The result is 5.9 ± 0.5 ns for rhodamine 6G and 2.9 ± 0.3 ns for pyronine G. Data reduction was carried out on an Apple II microcomputer in both cases.

4. Discussion

The main aim of the present work is not to introduce an alternative experimental method for estimating the fluorescence decay time of the dyes, but to demonstrate the potential possibilities of fluorescence correlation spectroscopy in the studies of rotational diffusion and intramolecular dynamics in solutions up to the nanosecond time range. It should be noted, however, that the necessarily modest signal-to-noise ratio indicates the need for careful design of both the apparatus and the photochemical aspects of further experiments. At the same time, the ability to study the dynamics of even very few fluorescent centres may provide new possibilities in solid state physics as well as biophysics.

References

Ehrenberg M, Rigler R (1974) Rotational brownian motion and fluorescence intensity fluctuations. Chem Phys 4: 390–401

Elson EL, Magde D (1974) Fluorescence correlation spectroscopy. I. Conceptual basis and theory Biopolymers 13:1–27

Kask P, Kändler T, Piksarv P, Pooga M, Lippmaa E (1983) Fluorescence correlation spectroscopy in solutions. In: Springer Ser Opt Sci 38 (Photon correlation techniques in fluid mechanics): 393–398

Kändler T, Kask P, Piksarv P, Sirk A, Lippmaa E (1982) Chemical kinetic studies by fluorescence correlation spectroscopy Eesti NSV Teaduste Akadeemia Toimetised Füüsika Matemaatika 31:314–319

Kimble HJ, Dagenais M, Mandel L (1977) Photon antibunching in resonance fluorescence. Phys Rev Lett 39: 691–695

Koppel DE (1974) Statistical accuracy in fluorescence correlation spectroscopy. Phys Rev A10:1938–1945

Koppel DE, Axelrod D, Schlessinger J, Elson EL, Webb WW (1976) Dynamics of fluorescence marker concentration as a probe of mobility. Biophys J 16:1315–1329

Kozierowski M (1981) Photon antibunching in nonlinear optical processes. Kvantovaja Elektron. (Moscow) 8: 1157–1169

Leskovar B, Lo CC, Hartig PR, Sauer K (1976) Photon counting system for subnanosecond fluorescence lifetime measurements. Rev Sci Instrum 47:1113–1121

Magde D, Elson EL, Webb WW (1974) Fluorescence correlation spectroscopy. II. An experimental realization Biopolymers 13:29–61

Rigler R, Grasselli P, Ehrenberg M (1979) Fluorescence correlation spectroscopy and application to the study of brownian motion of biopolymers. Phys Scr 19:486–490

Steinberg IZ, Haas E (1982) Intramolecular dynamics of polymer molecules studied by long-range nonradiative energy transfer. Jerusalem Symp Quantum Chem Biochem 15 (Intramol Dyn):459–472