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

Submillisecond Detection of Single Rhodamine Molecules in Water[1]

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

Using a modified confocal fluorescence microscope and a CW argon laser, we have measured fluorescence bursts from diffusing single Rh6G molecules that clearly exceed the background intensity. The exact average number of molecules in the observable volume element was measured directly via the fluorescence intensity autocorrelation function. This allowed us to estimate the probability of finding several molecules simultaneously in the volume element. A tradeoff between the number of detected fluorescence photons and the signal-to-background ratio was observed. In a volume element of 0.24 fl, 4 photoelectrons on average were detected from a molecule of Rh6G with a fluorescence-to-background ratio of 1000, while the volume element of 60 fl yielded on average 100 photoelectrons with a background of 25 counts. In fast single-molecule detection the intersystem crossing into the triplet state plays an important role, affecting the maximum emission rate from the molecule.

KEY WORDS: Fluorescence; single molecules; rhodamine 6G; autocorrelation; CW.

INTRODUCTION

The possibility to study single fluorescent molecules in the solid state [1,2] and in liquids [3,4] has been demonstrated recently. Single-molecule detection in water solutions can have important applications in the analysis of trace compounds and rare events in chemical and biological systems. Compared to solid matrices at temperatures below 2 K, single-molecule detection in solution at room temperature poses several additional difficulties:

(1) The absorption cross section is smaller by more than four orders of magnitude;

(2) The molecule emits only a limited number of photons before it is photobleached (on average 25,000 for Rh6G in water); and

(3) The molecules are undergoing continuous Brownian motion, making their manipulation very difficult and limiting the observation time.

The difference in absorption cross sections is caused by inhomogeneous spectral line broadening, which at 2 K is static, and lasers can be tuned into resonance with the zero-phonon absorption line of a single molecule. At room temperature the absorption lines fluctuate over the whole inhomogeneous line width according to changes in surroundings of the molecules, resulting in significantly reduced time-average absorption. The common obstacle for both solution and solid state is that the background radiation (Rayleigh and Raman scattering, fluorescence of impurities) and its noise tend to hide the weak fluorescence of single molecules. Single rhodamine molecules have been detected in ethanol and water solutions [3], using time-gating with picosecond lasers

[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.

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1053-0509/94/0900-0259$07.00/0

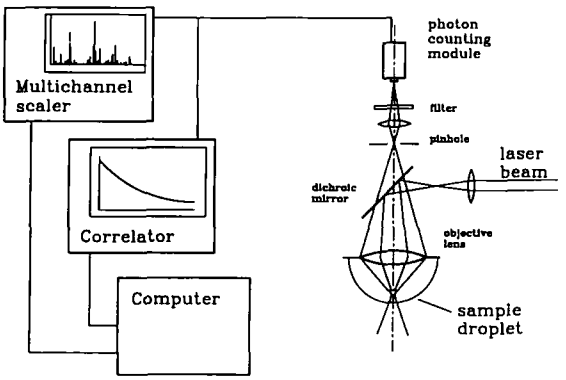

Fig. 1. The setup for detecting single molecules. For excitation a CW argon ion laser, operating at 514.5 nm, is used. The interference filter (Omega Optics 565DF50) removes the Rayleigh scattered laser light and the strongest band of the Raman scattering of water at 3200–3600 cm^{-1}. The fluorescence is collected by a 63×1.2 water-immersion objective and detected by an avalanche photodiode with quantum efficiency of 40% at 600 nm (EG&G Optoelectronics Canada SPCM-100). The dimensions of the observable volume element are defined by the diameter of the focused laser beam in the sample droplet and by the pinhole in the image plane.

and time-correlated single-photon counting to discriminate against the background light. We show here that the signal-to-background ratio in the case of CW excitation can be improved considerably by appropriate reduction of the Raman scattering of the solvent.

THEORETICAL BACKGROUND

Autocorrelation Function

The observed fluorescence signal can be quantitatively characterized using the intensity autocorrelation function $G(\tau) = \langle I(t)I(t+\tau)\rangle/\langle I\rangle^2$. In the case of molecules diffusing freely in and out of the observable volume element with a three-dimensional Gaussian intensity distribution, the normalized autocorrelation function has the following form [5], with a correction for the background intensity I_b [6]:

$$G(\tau) = 1 + \left(1 - \frac{I_b}{I}\right)^2 \Big/ N\left(1 + \frac{4D\tau}{w_0^2}\right)\sqrt{1 + \frac{4D\tau}{z_0^2}} \quad (1)$$

where w_0 is the radius of the volume element, $2z_0$ is its length, D is the translational diffusion coefficient of dye molecules, N is the average number of dye molecules in the volume element, I_b is the background intensity, and I is the observed intensity during the measurement.

In a confocal microscope the observed intensity profile along the laser beam is not Gaussian, but two-piece Lorentzian, as determined by the focused laser beam and the effect of the pinhole in the image plane. However, due to the small contribution of the z-dimension to the correlation function, expression (1) can be used as a very good approximation [7]. From the autocorrelation function [Eq. (1)] two important parameters can be directly evaluated:

(1) The average number of molecules in the observable volume element and
(2) The average residence time of the molecules in the volume element, which is roughly equal to $\tau_{diff} \approx w_0^2/4D$, as usually $z_0 > w_0$.

Signal-to-Background Ratio

In single-molecule detection, the highest possible fluorescence emission rate is desirable, simultaneously with a high signal-to-background (S/B) ratio. At very high excitation intensities the fluorescence saturates, while the background is always proportional to the excitation, leading to a reduced signal-to-background ratio. The optimal (presaturation) intensity of the exciting laser light is defined by the photophysical properties of the dye molecules in solution and should be kept constant when changing the laser beam size. Under this condition the intensity of Raman scattering I_R is proportional to the number of water molecules in the volume element: $I_R \sim w_0^3$.

The useful counting interval is somewhat larger than the average residence time in the volume element; we have used $t_c = w_0^2/2D$.

The number of background counts B during the counting interval is $B = t_c \times I_R$, which is proportional to w_0^5.

In the case of a small volume element, when most of the molecules diffuse out of the laser beam before they are photobleached, the number of signal counts S is proportional to t_c, and therefore S/B $\sim 1/w_0^3$.

The photocount number distribution is a useful characteristic of the single-molecule fluorescence record, allowing the analysis of detection probabilities and the effects of multimolecular events. Assuming negligible photobleaching and random diffusion, this distribution can be approximated by the spatial distribution of the intensity in the volume element [4].

EXPERIMENTAL

The experimental setup (Fig. 1) was as described before [7] with two modifications: A new correlator with

Table I. Comparison of Signal-to-Background Ratio for Different Filters and Volume Element Sizes[a]

Filters	Signal [counts/ (molecule×sec)]	Background (counts/s)	S/B	τ_{diff}(μs)	Counts/ diff. time	Laser power (mW)	w_0 (μm)
Glass cutoff	100,000	2,500	40	40	4	0.25	0.26
Interference							
Narrow	35,000	20	1,700	40	1.4	0.25	0.26
Narrow	30,000	200	150	140	4.2	1	0.5
Narrow	30,000	3,000	10	1,000	30	10	1.6
Wide	100,000	100	1,000	40	4	0.25	0.26
Wide	100,000	25,000	4	1,000	100	10	1.6

[a]Signal means the average fluorescence intensity of a single molecule of Rhodamine 6G. The configuration with S/B = 1700 cannot be used for single-molecule detection because of the low number of counts per diffusion time.

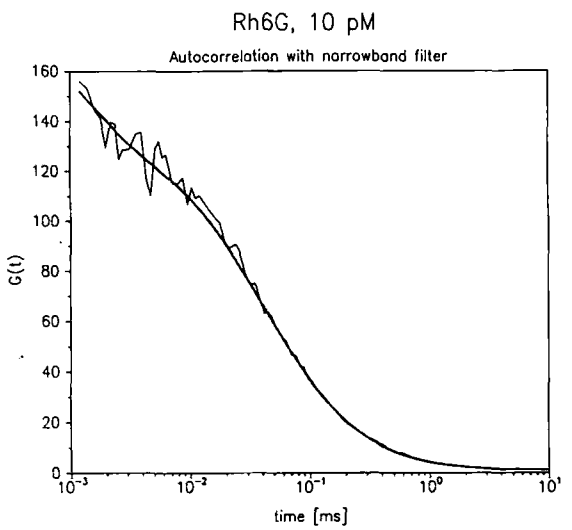

Fig. 2. Autocorrelation function for 10^{-11} M rhodamine 6G. In this case the fluorescence intensity equals the background intensity and $N = 1/4[G(0)-1] = 1/600$. The diffusion time $\tau_{dif} = 40$ μs. Triplet-state correlation time $\tau_{tr} = 1.3$ μs, and population of the triplet state $f_{tr} = 26\%$, meaning that molecules spend 26% of the time in the triplet state. The curve-fitting procedure is described in Ref. 9.

a logarithmic time scale (ALV-5000) and a multichannel scaler (Tennelec MCS II) were used. The diameter of the beam focus was adjusted by varying the laser beam size at the entrance lens. The laser power was adjusted to achieve the peak intensity of 250 kW/cm² in the sample. The solution was placed directly on the water immersion objective as a hanging droplet. With larger volume element sizes the contamination of the objective with rhodamine can become a problem. In this case a coverslip was used between the specially corrected objective and the sample droplet. The solutions were prepared in Millipore MilliQ purified water by diluting from a 10^{-5} M stock solution.

RESULTS

The single-molecule fluorescence (signal) and background data for different volume element sizes and emission filters are presented in Table I. The signal-to-background (S/B) ratio follows reasonably well the $1/w_0^3$ relationship. Using the bandpass filters that block the strongest Raman band of water at 3400 cm^{-1}, instead of a simple long-pass filter, we achieved a significant reduction of the background intensity, improving the S/B ratio from 40 [4] to 1000. The narrow-band (550 to 570-nm) filter, although yielding the highest S/B ratio, was less suitable for single-molecule work due to its reduced transmittance (50% max). The experimental autocorrelation function in Fig. 2 with its high amplitude, illustrates the high S/B ratio.

The photoelectron bursts from single molecules (Figs. 3 and 4) were recorded using a wide-band filter (545–595 nm) with a peak transmission of 95%. Due to the high S/B ratio with a volume element of 0.24 fl (Fig. 3), the molecules could be driven into saturation with no significant increase in background counts. This feature allows one also to detect molecules with lower absorbances than rhodamine 6G. With an average background count number of 0.01, only four "signal" photoelectrons are needed to detect a molecule with a reasonably low error rate of 4×10^{-6} s^{-1}. Such a low threshold, corresponding to fewer than 100 emitted photons, facilitates detecting photoliable molecules. The importance of a low threshold follows from the fact that the probability density distribution of the number of photons emitted from a single fluorophore is a decaying exponential [8].

In the 60-fl volume element (Fig. 4) the S/B ratio was only 4, but due to better counting statistics, the bursts are clearly distinguishable. However, a consider-

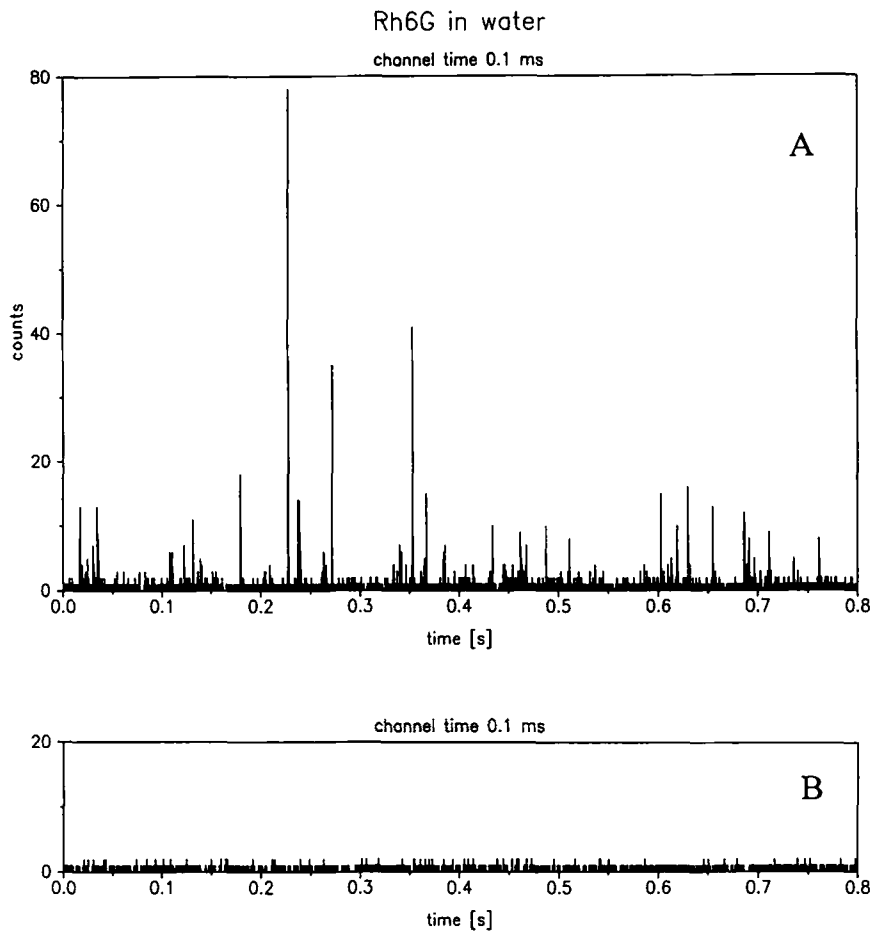

Fig. 3. Multichannel counter traces for (A) 2×10^{-10} M rhodamine and 6G and (B) a pure water background. Channel time was 100 μs. Radius of the volume element w_0 = 0.26 μm; power of the laser beam P = 1.4 mW. In this case only four photocounts are needed for detecting the presence of a molecule, corresponding to approximately 80–100 emitted photons.

ably larger fraction of the molecules is photobleached before exceeding the background count number. For just detecting the molecules or for counting them, the configuration with the lowest background is preferable. If the purpose is to obtain more detailed information (spectral contents of the emission, lifetime of the excited state), then a larger number of photons is needed and one has to consider that a higher fraction of molecules will be lost due to photobleaching.

The experimental and simulated distributions of photocount numbers (Fig. 5) for the traces in Fig. 4 show that for the majority of the burst amplitudes, the single-molecular events are dominating. Only for bursts of more than 1100 counts does that probability of two-molecular events become higher. This strong contribution from two-molecular events was caused by the relatively high concentration of Rh6G in this experiment (the probability of finding a molecule in the volume element during the 4-ms counting interval was 0.3).

An important limitation in single-molecule detection is the intersystem crossing from the singlet state into the nonfluorescent triplet state, due to the long lifetime of the latter. Depending on the degree of triplet population buildup, fast detection may become impossible. The effect of the triplet state becomes observable when the intersystem crossing rate K_{ISC} approaches the triplet decay rate K_T, as the saturation emission rate is proportional to $K_T/(K_{ISC} + K_T)$. Knowledge of these parameters is useful in selecting fluorophores for single-molecule detection. A detailed study of the triplet formation kinetics of rhodamine 6G has been done by Widengren *et al.* [9].

The background can be significantly reduced by using pulsed excitation and time-gated detection, due to

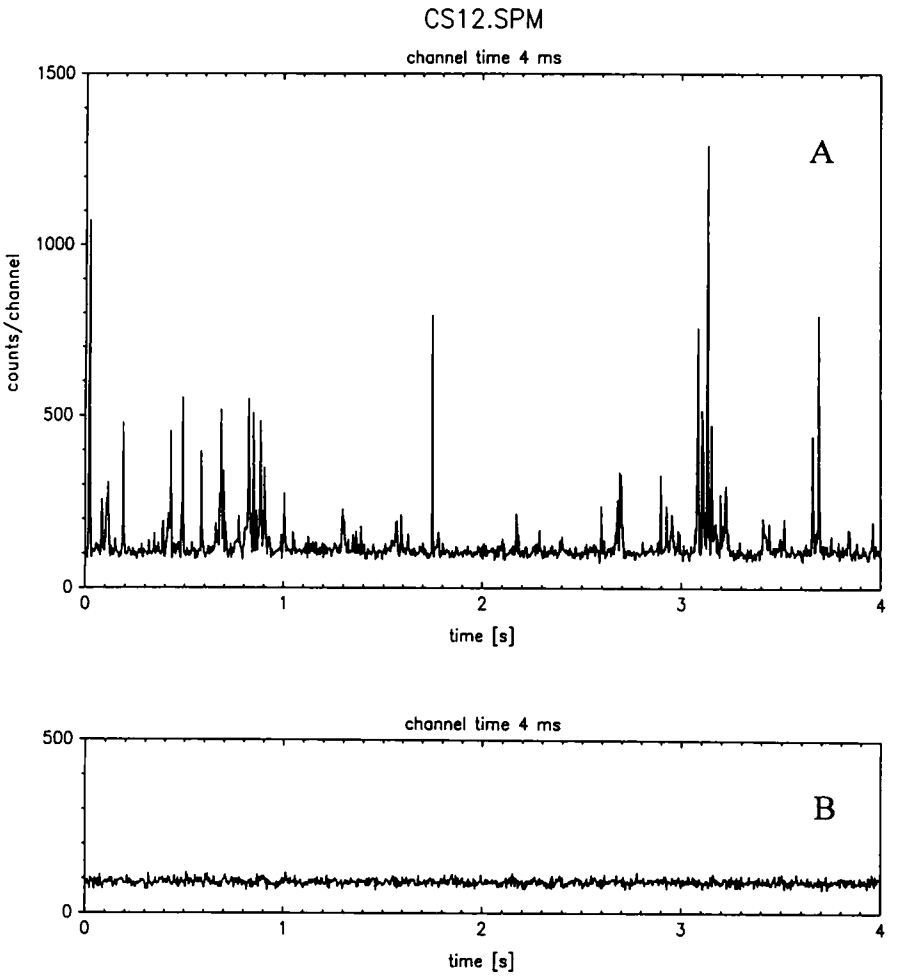

Fig. 4. Multichannel counter traces for (A) 2×10^{-12} M rhodamine 6G and (B) a pure water background. Radius of the volume element w_0 = 1.6 μm; power of the laser beam P = 10 mW. Due to the much higher background here, only the bursts of more than about 100 photoelectrons can be clearly identified. The bursts of 800–1000 photoelectrons correspond to about 20,000 emitted photons, which is close to the average number before photobleaching for rhodamine 6G in water.

the instantaneous nature of Raman scattering [3]. Based on time-correlated single-photon counting involving time-to-amplitude converter, this method suffers from dead times of several microseconds and is useful only when each excitation pulse generates much less than one detected Raman photon. Also, the excitation rate is limited to the laser pulse frequency, and the equipment is rather complex.

CONCLUSIONS

(1) Single molecules with absorption cross sections and quantum yields such as rhodamines can be detected in water with CW excitation at time intervals of 100 μs. The high signal-to-background ratio (1000:1) will enable detection of molecules with absorbances and quantum yields lower than those of rhodamine 6G.

(2) By increasing the volume element of observation, the number of photons collected from one rhodamine 6G molecule can be increased to several hundred, and spectroscopic analysis will be possible.

(3) CW laser excitation for single-molecule detection, while suffering from a higher background, has some significant advantages over time gating with time-correlated photon counting: (i) shorter dead times, resulting in fewer photons lost; (ii) higher excitation rates, leading to increased photon rates emitted by a molecule; and (iii) considerably simpler equipment.

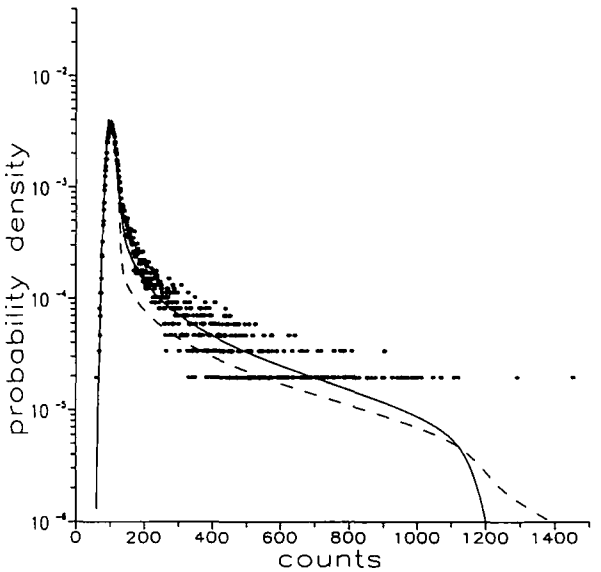

Fig. 5. Photocount number distributions for the experiment in Fig. 4. (•••) Experimental values; (——) calculated distribution for single molecules; (---) calculated distribution for two-molecular events.

(4) Due to the low background and high fluorescence detection efficiency, very few emitted photons (less than 100) are needed to detect a rhodamine molecule.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Technical Science Research Council, L. and J. Grönberg Foundation, and the Karolinska Institute.

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