Submillisecond Detection of Single Rhodamine Molecules in Water

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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...
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]:

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

(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 $\tau_{\text{diff}} = w_0^2/4D$, 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: $I_R \sim w_0^2$.

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 $w_0^4$.

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 $S/B \sim 1/w_0^4$.

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