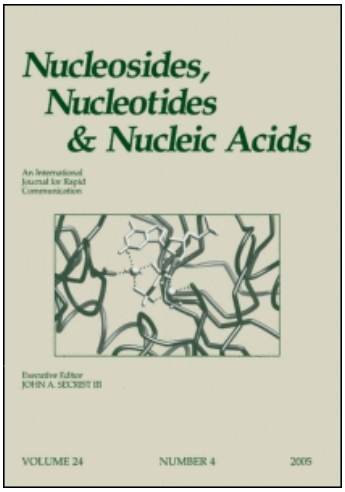

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Single-Molecule Detection of Coumarin-120

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SINGLE-MOLECULE DETECTION OF COUMARIN-120

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ABSTRACT
Two-photon excitation with a mode-locked titanium: sapphire laser at 700 nm and confocal fluorescence microscopy have been used to detect single Coumarin-120 (C-120) molecules. The dye C-120 is quenched by the nucleobases, if coupled to nucleotides, resulting in nucleobase-specific fluorescence lifetimes. This suggests applications in current projects for ultrasenstive DNA characterization.

Efficiency of one-photon and two-photon excitation

Detection of single molecules by laser-induced fluorescence (LIF) opens new horizons for applications in analytical chemistry, biology and medicine [1]. An important class of dyes, used as fluorescent labels in life sciences (e.g. AMCA), are coumarins [2]. As shown in FIG.1, the dye Coumarin-120 is quenched by the nucleobases, if coupled to nucleotides, resulting in nucleobase-specific fluorescence lifetimes ranging from 1.4 ns to 5.3 ns [3]. This suggests applications in current projects for ultrasenstive DNA characterization.

In previous studies of single molecules in liquids by one-photon excitation (OPE), small detection volumes V in the range of 1 pl to 1 fl were used to reduce the background signal caused by the Raman emission of water, which increases proportional to detection volume [4]. The limitation for the size of the detection volume can be circumvented by two-photon excitation (TPE) of the fluorophore in the NIR, because Rayleigh as well as Raman scattered photons are well separated spectrally from the fluorescence signal occuring in the UV/VIS. In TPE experiments for SMD the size of detection volume is in principle unlimited. However, a useful size depends on several factors: I.) concentration of the fluorescent dye; II.) fluorescence background due to impurities; III.) diffusional transit time, in which an average number of fluorescence photons is emitted; and IV.) photobleaching. TPE has been used successfully for SMD of Rhodamine B in water [5],

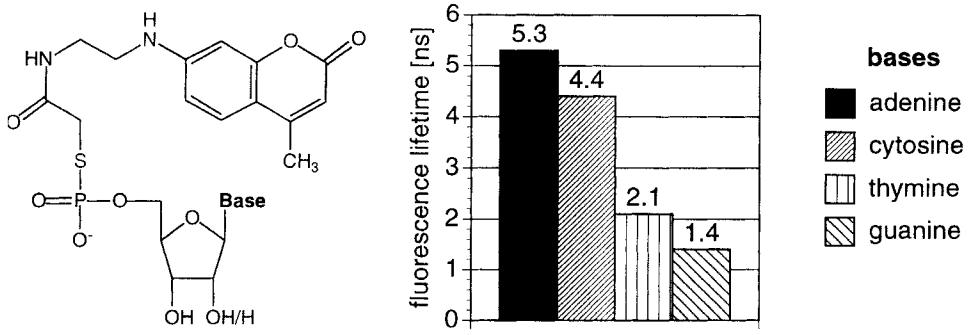

Figure 1. Fluorescence lifetimes of conjugates C-120/N between C-120 and nucleotides.

SMD in solids [6], fluorescence correlation spectroscopy in cells [7] as well as in fluorescence microscopy [8].

As a first step, we present in the present communication results on the efficiency of SMD of Coumarin-120 by TPE in solution using fluorescence correlation spectroscopy and time-resolved fluorescence spectroscopy. Coumarins are ideal candidates for SMD by TPE, because they have high TPE cross sections of approximately 20×10^{-50} (cm^4 s) in the wavelength region about 700 nm.

The quadratic intensity dependence of the TPE fluorescence was investigated on a 5×10^{-8} M aqueous solution of C-120 with an excitation wavelength of 700 nm. In FIG.2A the fluorescence photon flow F(t) is plotted versus mean peak photon flow $E_{P,pk}$. Three characteristic phases are observed. I.) If the data are fitted according to $y = ax^{\beta}$ for a small photon flux, we obtain the expected value $\beta = 1.99$. The fluorescence decay curves are single exponential with the typical fluorescence lifetime of 5.0 ns for C-120 in water [3]; II.) Saturation of fluorescence begins to occur at an photon flux of 1.5×10^{30} photons cm^{-2} s^{-1}; III.) If the photon flux is greater than 1×10^{31} photons cm^{-2} s^{-1}, the signal decay curves show a new component with a fast decay time (open circles in FIG.2a) and the fluorescence rate decreases. We assign the signal to continuum generation induced by a combination of several intensity effects.

For comparison, FIG.2B shows the efficiency of OPE for C-120, which has an absorption maximum of the S_0 -S_1 transition at 341 nm in water. We could show by transient absorption spectroscopy that the S_1 as well as the T_1 state absorb in the same wavelength region making a multi-photon photolysis on UV excitation possible. Plotting the fluorescence photon flow F(t) versus mean peak photon flow $E_{P,pk}$, the data are fitted according to $y = ax^{\beta}$ for a small photon flux. The obtained value $\beta = 0.66$ is considerably smaller than the expected value of 1 indicating significant photobleaching by two-photon

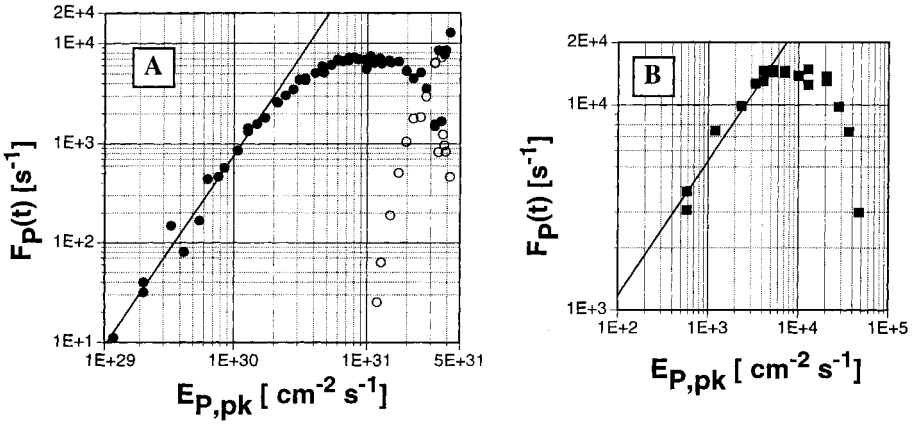

Figure 2. Average fluorescence flow F(t) for TPE (**A**) and OPE (**B**) is plotted versus the mean peak photon flux $E_{P,pk}$ (pulse width 300 fs, frequency 76 MHz). **A**: Excitation wavelength of 700 nm, Coumarin-120 , 5×10^{-8} M in water (filled circles) and continuum light (open circles) for water, detection volume V ≈ 1 fl. **B** :Coumarin-120 , 1×10^{-9} M in water , V ≈ 100 fl.

ionisation. This becomes also evident by the dramatic decrease of the fluorescence flow at a higher laser photon flux.

To conclude, TPE fluorescence of C-120 is limited by competing nonlinear effects. Saturation occurs also for the TPE fluorescence of Rhodamine 6G and Rhodamine B under the same conditions (data not shown). On the other hand, considerable photobleaching due to two-photon ionisation occurs for OPE leading to low fidelity for applications with single-molecule detection. Even if the efficiency of OPE is five to ten times higher than TPE [9], we choosed TPE to detect single C-120 molecules, because the photostabilty of C-120 was found to be sufficiently high under this conditions.

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) was used as a tool to obtain precise statistical characteristics with respect to an average molecule number in the detection volume and an average diffusion time [10]. The time dependent fluctuations of the fluorescence photon flow $F_P(t)$ were analyzed by the normalized intensity autocorrelation function $G(\tau_c)$, where τ_c is the correlation time. If translational diffusion is the only noticeable process that causes the measured fluorescence flow $F_P(t)$ to fluctuate about the average fluorescence flow $\langle F_P(t) \rangle$, $G(\tau_c)$ is given by Eq.1 [10].

$$G(\tau_c) = \frac{\langle F_P(t+\tau_c) F_P(t) \rangle}{\langle F_P(t) \rangle^2} \qquad \text{Eq.1}$$

$$= 1 + \frac{(1 - I_B/S)^2}{\sqrt{8}N} \left(\frac{1}{1 + (\tau_c/\tau_{D2})} \right) \left(\frac{1}{1 + (\omega_0/z_0)^2 \times (\tau_c/\tau_{D2})} \right)^{1/2}$$

The ratio of background intensity I_B to the total signal intensity S (S= F + I_B) is included in Eq.1 [10b] in order to correct the decrease of amplitude G(0) caused by the background signal. N is the average number of molecules in the detection volume and τ_{D2} is the characteristic time for diffusion in the excitation volume. The detected two-photon fluorescence volume is described by a squared three-dimensional Gaussian one-photon intensity distribution (radial $1/e^2$ radius ω_0, axial $1/e^2$ radius z_0). ω_0 is related to τ_{D2} via the translational diffusion coefficient D: $\tau_{D2}=\omega_0^2/ 8D$.

A typical normalized fluorescence correlation function of 10^{-11}M C-120 solution is shown in FIG.3. Using a diffusion coefficient D= 8×10^{-6} cm^2/s for C-120 in water and the results of the fit, given in Fig.3, a detection volume V of 0.95 fl is calculated. Using V= 0.95 fl and a C-120 concentration of 10 pM, an average number of molecules in the detection volume $N_V = 5.7 \times 10^{-3}$ is calculated. The calculated value N_V is in satisfactory agreement with the value of N= 2.2×10^{-3}, obtained from the amplitude of autocorrelation function $G(\tau_c)$ (see Eq.1). These molecule numbers N give clear evidence for the single molecule events in our experiments.

Time-resolved fluorescence

We measured a sequence of 100 successive signal decay curves with a constant number of photons at a peak photon flow of 1×10^{31} cm^{-2} s^{-1} to obtain fluorescence lifetimes of single molecules. For analysis we have developed a MLE-algorithm with convolution [11, 12], which uses the entire signal for data analysis. A representative signal decay of C-120 in water and the corresponding instrument response function IRF are given in FIG.4A. The inset shows the surface for a minimal $2I_r$*-value [11], which determines the fluorescence lifetime τ and variable fractions γ of white light. The average number of photons, detected in a burst of C-120, was 30 for water. The distribution of the fluorescence lifetimes, derived from the individual bursts, is shown in FIG.4B. Fitting to a Gaussian distribution yields an average fluorescence lifetime τ_{av}=4.8 ns and a standard deviation of 1.2 ns This result is in good agreement with values obtained from separate measurements at higher concentrations as well as with literature data: τ=5.0 ns [3].

Conclusions

We have shown the characterization of a single fluorescent dye by time-resolved fluorescence spectroscopy with two-photon excitation due its characteristic fluorescence lifetime. Furthermore this appears to be the first single-molecule detection of a fluorescent dye, which has its one-photon S_0-S_1 absorption maximum in the near UV. A high

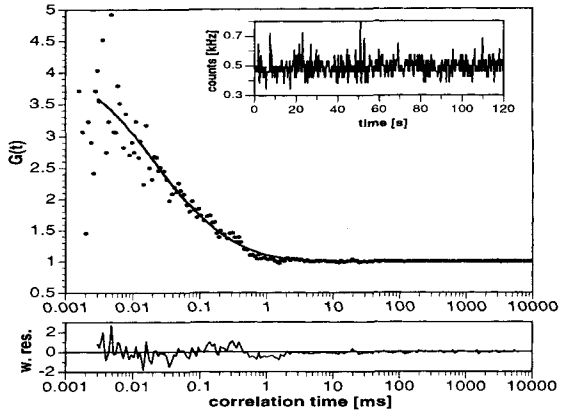

Figure 3. Normalized fluorescence autocorrelation function $G(\tau_c)$ for 10^{-11} M Coumarin-120 in water. Recorded data and fitted curve with weighted residuals [10], background intensity of 430 Hz and a signal intensity of 480 Hz. The obtained parameters: baseline: 0.9997, N: 2.2×10^{-3}, τ_{D2}: 43 μs (ω_0: 0.53 μm), z_0/ω_0: 8.5. The inset demonstrates the fluorescence bursts even on a s- time scale.

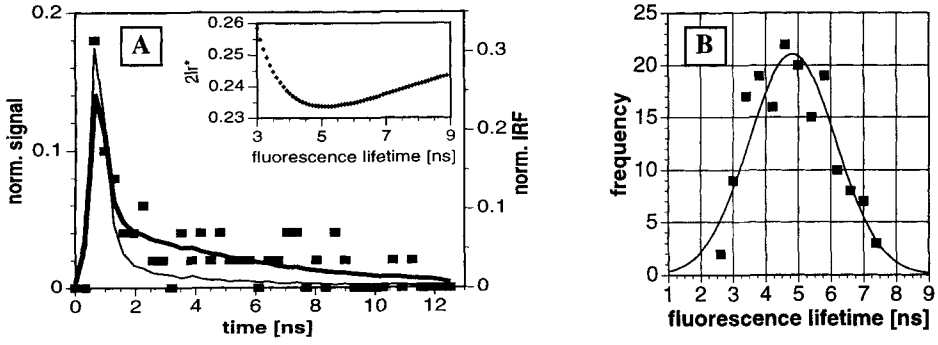

Figure 4. A: Normalized signal decay curves of bursts (left axis) of single Coumarin-120 molecules (10 pM solutions in water) Thin line: IRF, 5 channels of the peak were used for convolution,Bold line: Fitted curve obtained by a maximum likelihood estimator with a variable scatter fraction γ: τ= 5.1 ns; γ= 0.45. **B:** Fluorescence lifetimes τ obtained by the maximum likelihood estimator Fitting to a Gaussian distribution yields the following average fluorescence lifetime τ_{av}=4.8±1.2 ns

signal-to-noise ratio was achieved due to efficient rejection of background fluorescence and excitation light. TPE is free of multiphoton-photolysis and has a high sensitivity and selectivity due to the very high two-photon absorption cross-section of coumarins. This offers new possibilities for analytical applications of coumarins, when fluorophores of small molecular weight are needed.

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