Photonic mode density effects on single-molecule fluorescence blinking

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1. Silane functionalization of glass substrates

First, the glass substrates were cleaned with surfactant (Hellmanex-II, Hellma GmbH) and water followed by a treatment with a 1:1:5 mixture of H$_2$O$_2$:NH$_3$:H$_2$O at 80$^\circ$C for 30 minutes. 3-aminopropyltriethoxy-silane (3-APTES, Aldrich) was attached by immersion in a 0.1 M Milli-Q water solution of 3-APTES for one hour. Finally, the samples were rinsed with Milli-Q water, dried with N$_2$ and annealed for one hour at 120$^\circ$C.

2. Data analysis - $k_{21}$

$k_{21}$ was obtained from a single exponential fit to the histogram of the TCSPC micro-times (photon detection time after the last laser pulse). Figure 1 shows in a log-linear plot the histograms of the micro-times obtained from the direct light from the laser diode used in the experiments and the fluorescence decay of two different molecules. The system response was limited by the time response of the avalanche photodiodes.

![Figure 1. Histograms of the micro-times obtained from the laser diode (system response) and two different single molecules. All displayed curves were normalized and the time zero was placed at the first inflexion point to the left of the maximum. The fluorescence excited state lifetime ($1/k_{21}$) was obtained from single exponential fits.](image)

Faster detectors were not used because they do not provide the quantum efficiency necessary for single molecule detection. Nevertheless, simulations show that the method is reliable to obtain accurate lifetimes down to 0.6 ns.

3. Data analysis - $k_{31}$

Two methods were used for the analysis of the fluorescence blinking. The first method consists in generating a histogram of the TCSPC macro-times (photon detection times
from the beginning of the measurement) and using a suitable threshold to distinguish on- from off-times. An algorithm is used to find the optimum choice of bin-width and threshold (i.e. the best compromise between time resolution and accurate differentiation between on- and off-intensities). The algorithm needs as input the off-intensity which is directly measured and the on-intensity which can be obtained from a double exponential fit to a histogram of the inter-photon times (Fig. 2.a). Further details can be found elsewhere [1]. The off- and off-periods can then be identified as subsequent bins below and above the threshold.

\[ \tau_{on} = \frac{(k_{21} + k_{23})}{k_{12}k_{23}} \quad \tau_{off} = \frac{1}{k_{31}} \]  

For the case of triplet blinking those periods are exponentially distributed ((Fig. 2.c and 2.d)). According to the 3-level scheme shown in Fig. 1 of the paper, the average times of the off- and on-times distributions correspond to:

The other way to analyze the blinking is by means of the normalized intensity autocorrelation, which is defined as the rate of detection of two photons in a time interval
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$\tau$ relative to that rate if the photon detection would be uncorrelated. In practice, the TCSPC data is divided in time intervals of a given size $bw$ and the autocorrelation is calculated as:

$$C^{(2)}(\tau) = \frac{\langle N(t)N(t+\tau) \rangle}{\langle N(t) \rangle^2}$$

(2)

where $N(t)$ are the photons detected in a given interval (of duration $bw$) centered at a time $t$ and the angle brackets denote time average. For uncorrelated events $C^{(2)}(\tau) = 1$ and for correlated events $C^{(2)}(\tau) > 1$. If some physical process with a typical timescale $\tau_0$ is responsible for the correlation, a decay of $C^{(2)}(\tau)$ is observed around $\tau_0$, approaching unity for $\tau > \tau_0$.

From this decay it is possible to extract information about the dynamics of the process. For the fluctuations of the fluorescence emission of a single molecule due to excursions to the triplet state, the autocorrelation decay is a single exponential. Fig. 3 shows the normalized autocorrelation corresponding to the dataset shown in Fig. 2.

![Figure 3](image)

**Figure 3.** Normalized autocorrelation of the data set shown in Fig. 2. The red curve is a single exponential fit.

Based on the 3-level system, it is possible to obtain an analytical expression for this exponential decay [3, 2]:

$$C^{(2)}(\tau) = 1 + A e^{-\kappa \tau}$$

(3)

$$\kappa = k_{on} + k_{off}$$

(4)

$$A = \frac{k_{on} k_{off} (I_{on} - I_{off})^2}{(k_{on} I_{on} + k_{off} I_{off})^2}$$

(5)

where $k_{on} = 1/\tau_{off} = k_{31}$ and $k_{off} = 1/\tau_{on}$ are the rates of on- and off-switching, respectively, $I_{off}$ is the off-intensity equal to the experimental background and $I_{on}$ the on-intensity. Values for $A$ and $\kappa$ are obtained from the single exponential fit to the experimental autocorrelation (FIG. 3) from which $k_{off}$ can be obtained as:

$$k_{off} = \frac{\kappa A \langle I \rangle^2}{A \langle I \rangle^2 + (\langle I \rangle - I_{off})^2}$$

(6)
where $\langle I \rangle$ is the overall average intensity; i.e. the total number of detected photons divided by the total collection time. Then, $k_{on}$ can be obtained from EQ. 4.

**Figure 4.** Simulated fluorescence emission traces for different signal-to-background ratios $I_{on}/I_{off}$. In each case, the emission vs. time, the intensity distributions and the intensity autocorrelation are shown.

The reliability of these two analysis methods was tested by analyzing Monte-Carlo simulated data. The simulations are based on the 3-level system as described in the paper text. They take all the involved rates ($k_{12}, k_{21}, k_{23}$ and $k_{31}$) and the background intensity as input parameters and generate photon detection like the TCSPC module in
the experiments. Those simulated photon are then analyzed by means of the the trace-histogram and autocorrelation methods in the same way as the experimental data. The input parameters were adjusted in order to reproduce typical blinking of the investigated DiI molecules. A minimum number of on/off cycles is necessary in order to obtain reliable results from the analysis. For typical DiI blinking, this requires traces longer than 20 seconds [1]. 30 s traces were simulated with the same blinking statistics but different signal to background ratios; i.e. all the rates and the background intensity were kept constant and the on-intensity was intentionally reduced.

Figure 4 shows simulated traces at different $I_{on}/I_{off}$ ratios and their corresponding intensity distributions and normalized autocorrelation. As the $I_{on}/I_{off}$ ratio reduces, it becomes impossible to separate accurately the on- from the off-intensity in order to apply the trace histogram method. Though, the autocorrelation shows a clear decay down to very low SBR levels. Figure 5 shows the $k_{on}$ and $k_{off}$ obtained by means of the time-histogram and the autocorrelation methods as a function of the $I_{on}/I_{off}$ ratio. At high SBR both methods are of comparable accuracy and reliability; the autocorrelations shows a systematic deviation leading to slightly higher $k_{on}$ values, though. As the SBR reduces, the results obtained with the autocorrelation present a larger scatter but are still correct in their average. On the contrary, the trace-histogram method retrieves systematically too small values both for $k_{on}$ and for $k_{off}$.

![Figure 5](image_url)

**Figure 5.** $k_{on}$ and $k_{off}$ as a function of the $I_{on}/I_{off}$ ratio obtained by means of the time-histogram method and the autocorrelation from simulated data. The horizontal black lines indicate input values of $k_{on}$ and $k_{off}$ in the simulations. Each data point corresponds to the average and the error bars to the standard deviation of the results of 30 simulations with identical input parameters.
4. Correlation between $k_{31}$ and $k_{21}$

In order to test the significance of the observed correlations between $k_{31}$ and $k_{21}$, we determined the probability of obtaining the experimental $R$ values or higher from randomly ordered $k_{31}$ and $k_{21}$. Hence, the experimental $k_{31}$ and $k_{21}$ were randomly ordered in 100000 combinations and the $R$ for each combination was calculated.

![Figure 6](image-url)

**Figure 6.** Distributions of correlation coefficients $R$ between $k_{31}$ and $k_{21}$ obtained from $10^5$ random reorganizations of the experimental values of $k_{31}$ and $k_{21}$. The solid lines are Gaussian fits and the arrows indicate the experimental $R$ for molecules in samples I and II.

FIG. 6 presents the obtained distribution of $R$ from the $10^5$ random combinations of the experimental $k_{31}$ and $k_{21}$ of the molecules in samples I and II. The solid lines are Gaussian fits. The probability of obtaining $R$ values equal to or higher than the experimental one is $6 \times 10^{-5}$ for samples I and $3.5 \times 10^{-4}$ for samples II.

5. Independence of $k_{31}$ and $k_{21}$ of excitation intensity

In samples II, the gold film supports a surface plasmon polariton. Thus, the nearby gold film not only influences the de-excitation of the molecules but also their excitation rate due to the strong evanescent electric field of the surface plasmon. In order to assure that the observed enhancement of $k_{31}$ and $k_{21}$ is purely due to an increase PMD and not due to an increased excitation rate, $k_{31}$ and $k_{21}$ of molecules in samples I were measured under different excitation intensities. As it can be seen in FIG.7, there is no significant effect of the excitation intensity neither on $k_{31}$ nor $k_{21}$. Thus, effects of the excitation intensity can be ruled out.
Figure 7. Distributions of $k_{31}$ and $k_{21}$ obtained from molecules in samples I under three different excitation intensities; i.e. power of the excitation beam measured before the entrance into the microscope objective 1 $\mu$W, 3 $\mu$W and 7 $\mu$W.

References

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