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Fluorescence intensity and lifetime fluctuations of single Cy5 molecules immobilized on the glass surface

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Abstract

Single molecule fluorescence spectroscopy is a powerful tool to reveal the fluorescence fluctuation dynamics of a fluorescence molecule immobilized on a surface. These fluctuations are correlated in time under different excitation intensities with different repetition rate, which may provide the information for us to describe the reasonable excited state processes as well as the surrounding environments. In this report, we showed the first results on the fluorescence fluctuations of a single Cy5 molecule at the air–glass interface excited with different conditions by using far-field confocal laser microscopy in conjunction with multichannel scale (MCS) and time-correlated single photon counting (TCSPC). We found that the on/off-time and count rates showed different behaviors under different excitation modes. It was found that the excitation with the high repetition rate and/or strong intensity may cause high count rate, and increase the possibility of reverse intersystem crossing from T_n to S_n state, which may lead to longer on-time and shorter off-time. Furthermore, the photoinduced dynamic processes excited with strong peak power described by a five-level model under continuous wave (CW) and pulsed excitations with high repetition rate, it can be roughly simplified by a three-level scheme under pulse laser at lower repetition rate.

Keywords: Single molecule; Fluorescence fluctuation; Reverse intersystem crossing; On/off-time; Dynamic processes

1. Introduction

In the past few years, the detections and spectroscopies of single molecule have attracted great attention on the basic and applied research in the field of biology, physics and chemistry [1–4]. It is well known that single molecule detection can allow us to study molecular dynamic processes, such as spectral fluctuation, quantum jump, intensity fluctuation, photon bunching and photo antibunching, whereas in ensemble measurements, these phenomena are hidden [5–10].

The spectroscopic properties of a single molecule immobilized on and in thin polymer, gels and on glass surface have been widely studied at room temperature with different types of optical microscopes [11–14]. Since the fluorescence spectroscopy is one of the most sensitive methods for single molecule detection. The single molecules are studied through their fluorescence properties in which the most common study is the intensity fluctuation. The discrete intensity jump from high intensity (on-state) level to the background (off-state) is due to quantum jump of single molecule to the long-lived nonemissive dark state. This is called blinking, the typical on–off behavior of a single molecule, which has attracted a lot of interest [9,15–18]. Much work had been reported about the dynamic processes of a single molecule excited with CW and pulse lasers [15,19,20]. It was found that the typical three-level model is not always proper to describe the complicated excited state processes of single molecules.

Cy5, a well-characterized dye, is widely studied at single molecule level since Cy5 is one of the commercially available fluorescence dyes which can be used in near infrared wavelength range, and has a wide application especially in biological applications [21–23]. In order to study its dynamic behaviors under excitation at different conditions, we employ CW and pulse lasers to excite single Cy5 molecule. A reverse intersystem crossing is deduced from the fluorescence fluc-

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tuations, and a proper dynamic model is proposed to describe the excited state processes of single Cy5 molecules.

2. Experimental

In the experiment, a diode laser (PicoQuant) as pulsed excitation source (635 nm) and a He–Ne laser (632 nm) as CW excitation source were used to excite a single dye molecule. The excitation intensity described in our experiments means the incident peak intensity if without specific. The sample was prepared by spin-coating a droplet of 1 nM Cy5 (in ethanol) on the coverslip (20 mm × 20 mm) with 0.17 mm thickness. The laser light was led to the microscope through a single mode polarization maintain (SM PM) fiber. A 1/4 waveplate was used to change the polarized laser light into circular polarization light. An oil immersion objective (Olympus, 100×,1.4NA) was used both for focusing laser light onto sample and collecting the fluorescence from sample. The fluorescence that passed a dichroic mirror was focused onto a 100 μm pinhole for spatial filtering to reject out-of-focus signals and then reached the single photon avalanche diode (SPAD). For imaging, a 10 $\mu m^2 \times 10\ \mu m^2$ sample area was scanned through the objective. The fluorescence intensity time trace and lifetime were obtained by positioning the piezo scanner on one of the bright spot that was raster scanned. The fluorescence lifetime of single molecule was measured by time-correlated single photon counting with time-tagged time-resolved (TTTR) mode (TimeHarp 200, PicoQuant) with an instrumental response function of 500 ps (FWHM). All measurements were conducted in a dark compartment at room temperature.

3. Results and discussion

Fig. 1 displays the confocal fluorescence image of the single Cy5 molecule within a 5 μm × 5 μm area on the cover slip surface excited at 635 nm with two different excitation intensities (25 and 75 kW/cm^2 at 40 MHz). Each bright spot is attributed to fluorescence from one single Cy5 molecule. The full width at half maximum of typical spots is about 0.3 μm. The isolated dark pixels within the bright spot showed in Fig. 2 indicate that the fluorescence is not continuous in these spots. The dark periods that are different for different molecules are due to a temporal residence of the molecule in the triplet state. With a pixel integration of 0.6 ms, the maximal fluorescence intensity of a molecule 'a' in Fig. 2(a) is 6×10^3 counts, and is 40×10^3 counts in Fig. 2(b), which means that the excited probability of molecule is larger with stronger excitation intensity. Meanwhile, it is also found that the spots at different positions have different intensities. This variation among the molecules may be due to different molecular orientations and environments.

Fig. 2 shows the fluorescence decay of single Cy5 molecule on glass cover slip. The excitation intensity is about 25 kW/cm^2 at 40 MHz. The decay curve can be fitted with mono-exponential decay with the time constant about 2.0 ns. The lifetimes are distributed from 1.2 to 2.4 ns, which may depend on different dipole orientations, transition frequencies and dielectric constants of single molecules on the surface [13,24]. The average emission lifetime of single Cy5 molecules is about 2.1 ns. The fluorescence lifetime of single Cy5 molecule measured on the dry glass surface is longer than that about 1 ns measured in solution [22,23]. This may be due to decreasing flexibility of molecule on the dry surface.

Fig. 3 shows two different time traces from the same Cy5 molecule excited at different repetition rates with the same excitation intensity. The traces (a_1) and (a_2) are obtained at 10 and 40 MHz under the same peak power. The count rate histograms (b_1) and (b_2), on-time histograms (c_1) and (c_2) as well as off-time histograms (d_1) and (d_2) are extracted from (a_1) and (a_2), respectively. In order to investigate the excited state processes, the bin time for analysis of the duration histograms of molecule is always set to be 100 μs. The duration histograms of molecule are fitted with mono-exponential decay function in this paper, if there is no special specification.

From the on-time histograms (c_1), (c_2) and off-time histograms (d_1), (d_2), the on- and off-time can be obtained by mono-exponential fitting. When excited at 10 MHz, the

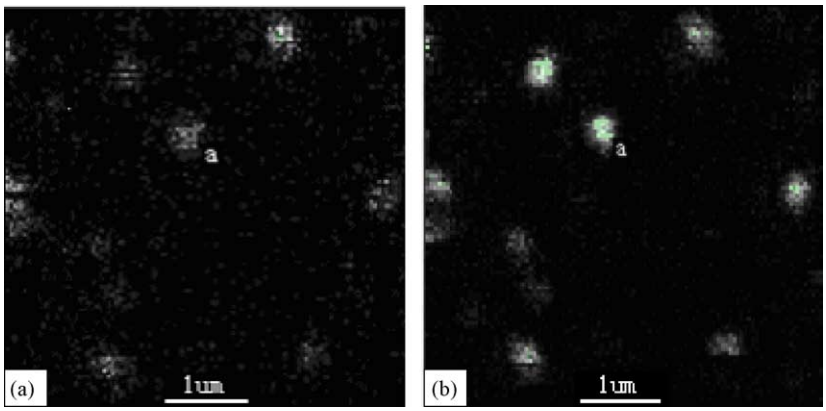

Fig. 1. Fluorescence image (5 μm × 5 μm) of single Cy5 molecule on the cover slip. The images are taken in 0.28 min (100 pixels × 100 pixels). The excitation intensity is 25 kW/cm^2 (a) and 75 kW/cm^2 (b), respectively.