Single-molecule detection using continuous wave excitation of two-photon fluorescence

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Two-photon fluorescence (TPF) is one of the most important discoveries for biological imaging. Although a cw laser is known to excite TPF, its application in TPF imaging has been very limited due to the perceived low efficiency of excitation. Here we directly excited fluorophores with an IR cw laser used for optical trapping and achieved single-molecule fluorescence sensitivity: discrete stepwise photobleaching of enhanced green fluorescent proteins was observed. The single-molecule fluorescence intensity analysis and on-time distribution strongly indicate that a cw laser can generate TPF detectable at the single-molecule level, and thus opens the door to single-molecule TPF imaging using cw lasers. © 2011 Optical Society of America

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Two-photon fluorescence (TPF) involves the simultaneous absorption of two photons, the combined energy of which is sufficient to bring the fluorophore to an excited electronic state [1]. The probability for two-photon excitation is proportional to the square of photon density. Consequently, TPF occurs most strongly near the optical focus within a limited volume of less than a femtoliter [2]. Because of the small localized excitation volume, both background fluorescence and photo damage are significantly reduced [3,4]. In addition, as TPF usually uses an IR laser for excitation, scattering and absorption are reduced compared to visible wavelength. It is therefore able to image thick samples with a good penetration depth [5–8]. Because of the intrinsic three-dimensional (3D) resolution, there is no need for confocal aperture in the detection path, which greatly simplifies the alignment of optics [9] and increases detection efficiency. However, to increase the efficiency of excitation at the laser focus, a pulsed laser is generally used for the imaging of biological samples [8]. Although a cw laser was shown to excite TPF [10,11] and was reported to excite a single quantum dot [12,13], its application in live cell imaging has been limited due to its perceived weak power [8].

Recently, we have constructed improved optical tweezers that allow detection of the angstrom level displacement using a biological-friendly 830 nm diode laser [14]. The choice of this laser wavelength allows TPF detection of enhanced green fluorescence proteins (EGFPs) in transfected mammalian cells [14]. To test the sensitivity of a cw laser-excited TPF using our optical tweezers setup, we developed a solution condition (150 mM NaCl, 100 mM phosphate buffer, pH 7.2) to immobilize purified EGFP molecules (98% purity, final concentration ~30 nM) nonspecifically on the inner surface of a homemade microfluidic chamber (Fig. 1). After a 30 min incubation, we applied a flow of phosphate buffer to remove excess unbound proteins. We then focused the cw laser beam precisely onto this EGFP-coated surface, scanned the microfluidic chamber laterally, and recorded fluorescence signals from the surface using an electron-multiplying CCD camera (Evolve, Photometrics). For all the imaging experiments, laser power was maintained at a stable level of 130.8 mW and exposure time was kept at 1 s.

During the scanning process, when the fluorescence signal increased abruptly above the background, it indicated that there were EGFP molecules in the laser focus. The scan was then immediately stopped and we recorded the fluorescence intensity in real time until it bleached (Fig. 2). Different types of fluorescence signals were observed. For 63% cases, the fluorescence intensity stayed at a relatively constant value, and then bleached [Fig. 2(a)], characteristic of the fluorescence emission from a single molecule followed by subsequent photobleaching [15,16]. Less frequently, the fluorescence intensity underwent two (34%) [Fig. 2(b)] or more apparent steps of decrease (3%) [Fig. 2(c) and 2(d)] before it bleached completely. No fluorescence signal was observed before we flew the EGFP solution into the chamber.

To quantitate these fluorescence signals, we used a t test analysis similar to previous methods [17] to detect

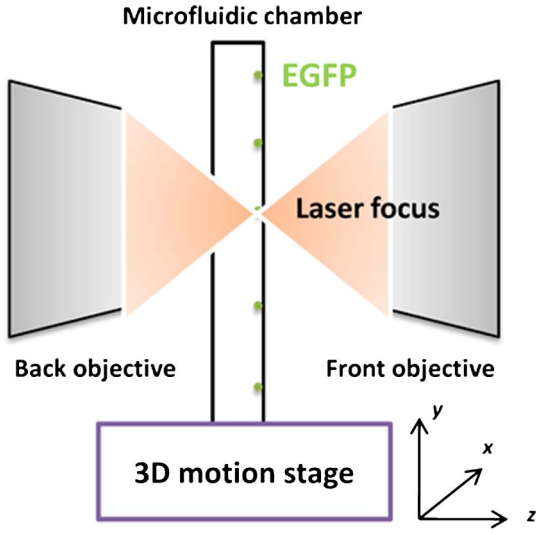

Fig. 1. (Color online) Experimental setup for single-molecule TPF imaging. Inner surface of the microfluidic chamber is sparsely coated with EGFP. The laser focus is placed precisely at the inner surface. Lateral scanning of the chamber driven by a 3D motion stage (ESP300, Newport) allows searching of EGFP molecules on the surface.

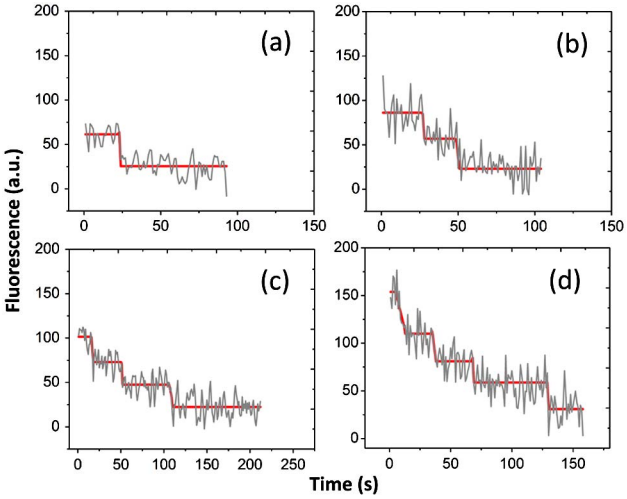

Fig. 2. (Color online) Fluorescence time courses of EGFP molecules immobilized on glass coverslips. Gray traces are fluorescence intensity data collected with 1 s exposure time for each data point. Red lines show the stepwise photobleaching of EGFP fluorescence detected and simulated by a custom-written Matlab program.

steps in these time trajectories of fluorescence, and obtained the histograms of fluorescence intensity and the on-time of fluorescence before photobleaching. As shown in Fig. 3(a), the histogram of fluorescence intensity show two major peaks, which can be well described by a double Gaussian function, with peaks centered at 33.3 ± 1.0 and 65.1 ± 1.0, respectively. The intensity value of 65.1 ± 1.0 is approximately twofold of the intensity value of 33.3 ± 1.0 within error, suggesting that under current illumination conditions, a single EGFP molecule contributes an intensity value around 33, and the intensity value around 65 is either contributed by an EGFP dimer [18] or two EGFP molecules that are closely l ocated within the laser focus. This intensity value for a single EGFP molecule converts to 9900 photons/s after consideration of our system collection efficiency (9%), the quantum efficiency of the camera chip, and system gains. Using the method developed by Schuck *et al.* [19], we further calculated the TPF cross section for EGFPs at 830 nm, which yielded a value of 24.8 GM and compared well with the recent report of 18.5 GM by Drobizhev *et al.* [20]. Figure 3(b) shows the distribution of the fluorescence on-time before photobleaching. Nonlinear least squares analysis shows that the data was best described by single exponential decay, with a time constant of 50.8 ± 4.2 s. This result suggests that the time to photobleaching for individual EGFP molecules was dominated by a single rate-limiting event, in agreement with the previous report [21]. Altogether, these data strongly indicate that the 830 nm cw laser used for optical trapping can excite fluorescence from EGFPs that is detectable at the single-molecule level. Each staircase in the fluorescence trajectories represents photobleaching of a single EGFP molecule.

One would expect a quadratic dependence of the fluorescence intensity on laser power if TPF occurs. To demonstrate that the fluorescence we detected from EGFP molecules indeed resulted from TPF, we measured EGFP fluorescence intensity as a function of the excitation laser

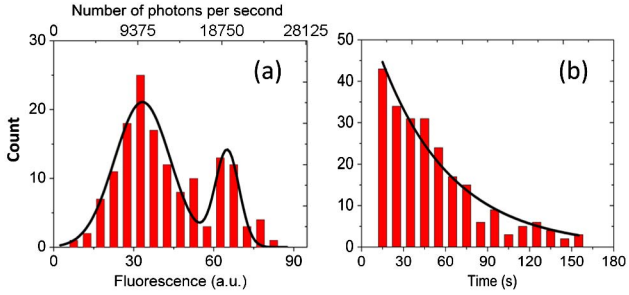

Fig. 3. (Color online) Statistics of single EGFP fluorescence. (a) Histogram of fluorescence intensity (bin size of 5 a.u.). The top x axis represents the number of photon counts per second. The black curve is fit by a double Gaussian function. (b) Histogram of fluorescence on-time before photobleaching (bin size of 10 s). The black curve is fit by a single exponential decay. The histograms were constructed based on the analysis of 176 fluorescence time traces represented by traces in Figs. 2(a) and 2(b).

power. We first conducted this experiment at the single-molecule level for EGFPs nonspecifically bound on the chamber surface (Fig. 1): we recorded fluorescence of single molecules before and after a quick increase of the excitation power [Fig. 4(a)] and found that the fluorescence intensity increased on average by a factor of 2.3 ± 0.4 when the laser power was increased by a factor of 1.57 (results from 35 single-molecule trajectories), consistent with two-photon excitation. Since single EGFP molecules are bleached with an average on-time less than 1 min, we coated polystyrene beads (1.3 μm diameter) nonspecifically with EGFPs and immobilized the bead on top of a micropipette to measure the fluorescence emitted from EGFP molecules on the bead surface in order to facilitate the continuous and systematic measurement of EGFP fluorescence as a function of laser power. Figure 4(b) inset shows the fluorescence image of such a polystyrene bead. Control experiments with uncoated beads showed no fluorescence. We measured the fluorescence intensity of the bead as a function of laser power and plotted them on a logarithmic scale in Fig. 4(b). These plots from different beads are all well described by a linear relationship, with an average slope of 1.89 ± 0.07. This value indicates that the fluorescence that we detected from EGFP molecules indeed resulted from TPF.

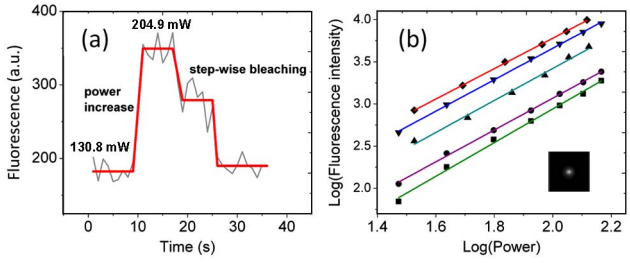

Fig. 4. (Color online) EGFP fluorescence intensity as a function of laser power. (a) At the single-molecule level, fluorescence intensity increases with increasing laser power and then bleaches in a stepwise manner. (b) Inset shows the TPF image of an EGFP-coated polystyrene bead (1.3 μm) immobilized on top of a micropipette. Different symbols represent measurements from different beads. Straight lines in different colors with different symbols show linear fits in a double logarithmic scale, which give an average slope of 1.89 ± 0.07.

In conclusion, we show that cw lasers can excite TPF that is detectable at the single-molecule level. Under current conditions, the signal-to-noise ratio is around 3 for 130 mW laser power. This power is equivalent to an average power of 0.70 mW from a regular pulsed laser (80 MHz, 200 fs pulse width). Since an average power of 0.2 mW from a pulsed laser is sufficient to allow TPF imaging of single molecules at room temperature [22], we expect this power is suitable for TPF detection at the single-molecule level. This amount of laser power is readily available from TEM00 diode lasers, and the signal-to-noise ratio of current TPF imaging can be further improved by using a laser at 900 nm, which is optimal for EGFP [23].

Stepwise photobleaching of single fluorescent molecules has become widely used to measure stoichiometry of macromolecules [24–28]. Our technique presented here is well suited for this purpose, and opens up the possibilities of using the cw laser as an alternative for TPF imaging and stoichiometry measurement in biological samples.

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