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Time-resolved single-molecule fluorescence microscopy: Pump–probe scheme employing bursts of pulses and gated photon counting

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ABSTRACT

A practical methodology to conduct the time-resolved fluorescence microscopy has been developed; the technique employs bursts of excitation pulses (≤ ms duration) gated by an electro-optic modulator from a 76 MHz ultrafast oscillator and enables incremental stage positioning along with gated photon counting in a pump–probe scheme. A fluorescence trajectory is thus spread into segments of the multiple scans, which advances an efficient data recovery not only over limited photon-cycles but a frequently blinking trajectory owing to the innate heterogeneity present in a single-molecule environment. A typical application that probes ultrafast dynamics of an interfacial electron transfer was demonstrated in a dye-sensitized TiO_2 nanoparticles at ambient condition.

1. Introduction

Recent applications of ultrafast spectroscopy to single molecule microscopy [1–4] open up a direct observation of the temporal inhomogeneity to structure–function relationship regarding electronic and chemical properties of molecules under a different micro-environment [5–7]. In the past decade, van Hulst and coworkers have developed single-molecule pump–probe techniques with an ultrashort (subpicosecond) time-resolution capable of tracking fast processes such as the intramolecular vibrational energy redistribution (IVR) in an individual molecule of its excited states [2,3]. Moreover, such a technical advance now spreads widely to observing single molecule coherence oscillations, [8] controlling vibrational wave packets, [9] and coherent energy transfer [10] in single molecular level at room temperature. However, those measurements might not be yet easy to conduct due to employing multi-color ultrashort pulses or the pulse-shaping technique needed to accommodate in pump–probe schemes.

Conventionally, the photon-counting technique is most probable to detect weak fluorescence from a single molecule and to best take a practical advantage of data acquisition using high rep-rate (tens of MHz) light sources, such as Ti:Sapphire (Ti:Sa)-based ultrafast oscillators widely available to date. With high rep-rate light sources used, a photon counting trajectory of a single molecule is typically completed within a few or tens of seconds due to the fact that its chromophore is likely photobleached after tens or hundreds of millions (10^7–10^8) photocycles. As such, it is unfavorable to incorporate such a way of single-molecule detection into conventional pump–probe schemes, because a translational stage takes a time to complete even a single scan in a short distance of movement. Most of the studies reported [1–7] thus far utilize an acousto-optic modulator (AOM) as a pulse picker to reduce the rep-rates down to 100–1,000 kHz such that the pump–probe schemes can be practically accommodated with continuous or multiple scans for a single-molecule detection to such a extended period of time.

In contrast to the 'sparse' pulse-trains scheme, this paper describes a practical alternative that employs 'bunches of excitation pulses' that are generated from tens of MHz pulse trains by an electro-optics modulator (EOM) with the capability of fast gating (≤ms). The excitation pulse sequence incorporates well in an incremental stage positioning followed by gated photon collection. What it follows is to apply for an interfacial electron transfer in dye-sensitized semiconductor nanoparticles, where fluorescence trajectory shows 'blink'; it repeats stochastic drops and recoveries until photo-bleached. The present technique demonstrates how to recover meaningful fluorescence depletion pump–probe microscopy under such blinking systems.

2. Experimental setup

In a confocal microscopic arrangement, the molecules studied were dispersed at concentrations of about 10^{-4} M and first raster-scanned to obtain a 2D fluorescence image map with a piezo-electric 2D-translational stage (Nano Bio-100-XY, Mad City Labs). An inverted confocal microscope (Observer.A1, Zeiss) comprised a plan-apochromatic, oil-immersion objective (63×) with a high numerical aperture (NA) 1.4, as illustrated in Fig. 1. The light source was generated from an optical parametric oscillator (Mira-OPO, Coherent/APE) that is employed with

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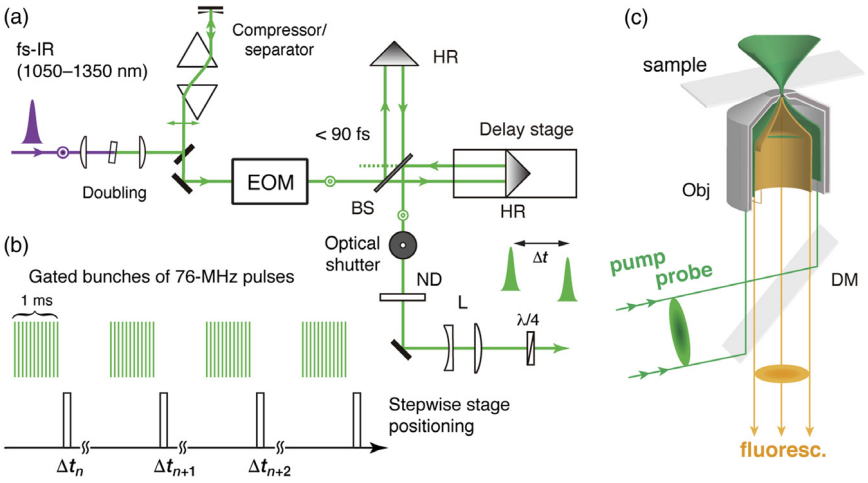

Fig. 1. Femtosecond time-resolved fluorescence microscopy with gated bunches of 76 MHz pulses: (a) an optical layout that consists of frequency-doubling unit followed by a prism compressor (< 90 fs), an electro-optic modulator (EOM) for pulse gating, and the Michelson interferometer to generate the pump–probe delayed pulses. (b) An incremental stage positioning along with both the bursts of excitation pulses and gated photon counting. (c) A schematic view of the inverted microscope used and its optical layout. See the text for details.

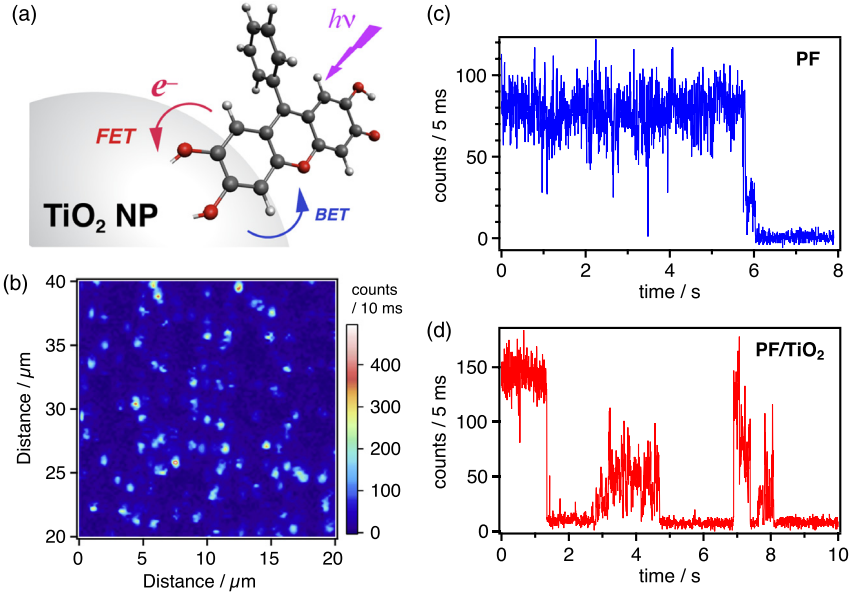

Fig. 2. The interfacial electron transfer from a dye molecule to semiconductor nanoparticles: (a) an example of a dye-sensitized system: photo-excited phenylfluorone (PF) molecule interacting with TiO_2 NPs. (b) 2D fluorescence image from spin-coated PF with TiO_2 NPs on a cover glass. The fluorescence trajectories of the PF molecule (c) alone and (d) with TiO_2, showing the fluorescence blinking in the presence of NPs.

a Ti:Sa femtosecond oscillator (Mira-900, Coherent; >1 W) pumped by a diode-pumped solid-state frequency-doubled Nd:YVO_4 laser (Verdi V-10, Coherent; 10 W). The typical OPO power was ≈350 mW and provided non-transform-limited pulses with a 250 fs pulse width in a 76 MHz pulse trains. After the OPO output was frequency-doubled via a thin BBO crystal (1 mm), the excitation wavelengths were in the visible range of 525–580 nm. A Brewster-angled prism-pair was used to compress the visible to transform-limited pulses of ~90 fs and separates the visible from the IR beam (not used).

An electro-optic modulator (M350-50: KD*P, ConOptics) was introduced to the beam path to serve as a fast optical shutter (≤ ms). As a result, the gated bunches of pulses (nearly 76k pulses per ms) were generated and synchronized to a gate-pulse to photon counting acquisition of the fluorescence. Effective overall rep-rate for the pulse bunches depends on how an incremental scanning is designed, but typically at 2–5 Hz, Fig. 1(b). The gated light was spatially filtered and collimated by a pair of lenses and a pinhole (ϕ = 20 μm) arrangement, led to a Michelson-type interferometer composed of a 50:50 beam splitter and a pair of 1.0-in hollow retroreflectors (UBBR1-2S, Newport). One of the retroreflectors was mounted on a motorized translational stage (MFA-CC, Newport), interfaced with a PC and controlled by a home-made software on LabVIEW platform (LabVIEW Pro 2014, NI). The pump and probe beams were generated through the interferometer, and their instrument response was evaluated by a home-made autocorrelator, resulting in a cross-correlation width of <130 fs (FWHM), consistent with the pump/probe pulse of <90 fs (FWHM).

The excitation beam, after passing through the interferometer and recombined collinearly, was directed into the microscope through a beam expander and a $\pi/4$ waveplate, and focused on the sample that was prepared by spin coat on a cover slip. To localize individual molecules, a 20 × 20 μm^2 region of the sample was scanned at a time by another home-built software with the LabVIEW. Spatially well-separated molecules were selected and successively moved into the focus of the objective for the pump–probe measurement. The fluorescence was