Nonlinear Fourier-transform spectroscopy using ultrabroadband femtosecond pulses for the measurement of photobleaching of fluorescent proteins

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Abstract: We examine the mechanism of photobleaching of fluorescent proteins using nonlinear Fourier-transform spectroscopy with ultrabroadband femtosecond pulses. Photobleaching of two-photon excited fluorescent molecules occurs through one-photon excited-state absorption.
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1. Introduction

Fluorescent protein is widely used as a tool in biological researches such as multi-photon fluorescent imaging [1]. However, photobleaching of fluorescent molecules is a crucial issue for correctly observing biological samples and for interpreting their molecular function. Although a number of experiments have been carried out to explore the mechanism of photobleaching, little is known about photobleaching in multiphoton processes.

We apply nonlinear Fourier-transform spectroscopy to the measurement of the action spectrum for photobleaching of fluorescent proteins using an ultrabroadband pulse with a spectrum ranging from 650 to 1200 nm. We show that sequential three-photon excitation process, excited-state absorption (ESA) following two-photon excitation (TPE) of fluorescent molecules, is one of the routes to photobleaching.

2. Nonlinear Fourier-transform spectroscopy

The principle of nonlinear Fourier-transform spectroscopy based on interferometric autocorrelation (IAC) measurements is shown as follows [2]. The second-order interferometric autocorrelation (IAC) signal $S^{(2)}(\tau)$ is given by

$$S^{(2)}(\tau)=\int_{-\infty}^{\infty}\left|\int_{-\infty}^{\infty}R^{(2)}(t-t_1)E(t_1,\tau)^2\,dt_1\right|^2 dt, \tag{1}$$

where τ, $R^{(2)}(t)$ and $E(t,\tau)$ are the delay time between two broadband pulses, the response function of the TPE process and the interferometric field, given by $E(t,\tau)=\left[A(t)+A(t-\tau)\exp(-i\omega_0\tau)\right]\exp(i\omega_0 t)$, respectively. To obtain the spectral information $\tilde{R}^{(2)}(\Omega)$, that is, the Fourier transform of $R^{(2)}(t)$, the power spectrum $\tilde{S}^{(2)}(\Omega)$ is calculated using the Fourier transform of $S^{(2)}(\tau)$. Then, the power spectrum at frequencies around $2\omega_0$ can be written as

$$\tilde{S}^{(2)}_{2\omega_0}(\Omega)=\left|\tilde{R}^{(2)}(\Omega)\right|^2\left|\tilde{A}^{(2)}(\Omega-2\omega_0)\right|^2, \tag{2}$$

where $|\tilde{A}^{(2)}(\Omega-2\omega_0)|^2$ is the second harmonic power spectrum obtained from a reference sample and $|\tilde{R}^{(2)}(\Omega)|^2$ is the TPE spectrum of a resonant sample. Therefore, the TPE spectrum can be acquired by dividing the second harmonic power spectrum of the resonant sample by that of the reference sample.

Assuming that photobleaching occurs through one-photon ESA following TPE from the ground state of the fluorescent molecules, the corresponding IAC signal $S^{(3)}_{PB}(\tau)$ can simply be expressed as

$$S^{(3)}_{PB}(\tau)=S^{(2)}_{TPE}(\tau)\cdot S^{(1)}_{ESA}(\tau) \tag{3}$$

where $S^{(2)}_{TPE}(\tau)$ and $S^{(1)}_{ESA}(\tau)$ are the IAC signals for TPE from the ground state and one-photon ESA, respectively. Since $S^{(3)}_{PB}(\tau)$ and $S^{(2)}_{TPE}(\tau)$ are obtained from the measurements of TPE with and without photobleaching, $S^{(1)}_{ESA}(\tau)$ can be obtained by dividing $S^{(3)}_{PB}(\tau)$ by $S^{(2)}_{TPE}(\tau)$. Finally, the ESA spectrum is calculated from the Fourier-transform of $S^{(1)}_{ESA}(\tau)$.

3. Experimental

We used a Ti:sapphire mode-locked laser with a spectrum ranging from 670 to 1100 nm as an excitation light source. We precompensated for dispersion of the broadband laser pulses caused by the optical elements, including the microscope objective lens, using a fused-silica prism pair and a liquid-crystal spatial light modulator placed in the Fourier plane in a 4f pulse shaper configuration. The prechirped pulses were sent to a Michelson interferometer. The output from the interferometer was focused within a fluorescent sample through the microscope objective lens. The sample used was enhanced Green Fluorescent Protein (eGFP). In order to prepare unbleached fluorescent molecules for each delay time, the sample set in a glass container, was translated using a three-dimensional electrical stage. The exposure time for each delay time was 200 ms.

Figure 1(a) and (b) show examples of the IAC traces for TPE and photobleaching of eGFP, respectively. The IAC trace for photobleaching was obtained from the reduction in the fluorescence signal due to photobleaching during the 200 ms irradiation for each delay time. Assuming that photobleaching is based on ESA following two-photon excitation, we divide the IAC trace for photobleaching by that for TPE, and then Fourier transform it to the spectral domain. Figure 2 shows the result of the ESA spectrum for eGFP together with the TPE spectrum. The ESA spectrum has a peak at around 700 nm. In a separate experiment, we found that this absorption corresponds to the $T_1 \rightarrow T_2$ transition after intersystem crossing.

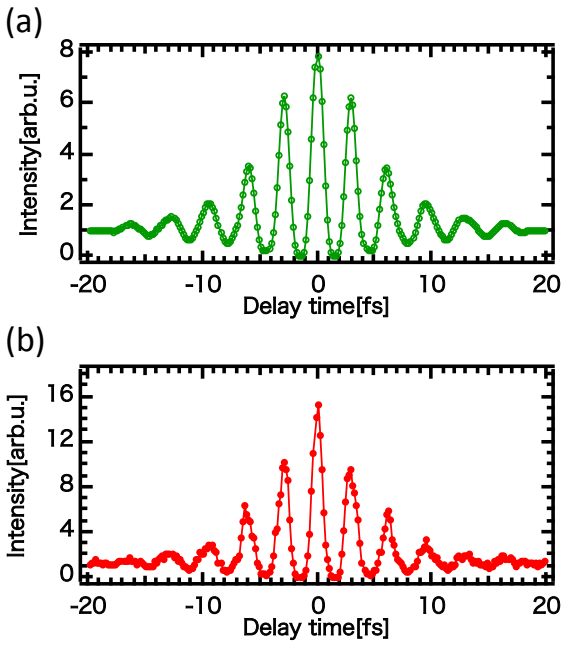

Fig.1 IAC traces for (a) TPE and (b) photobleaching of eGFP.

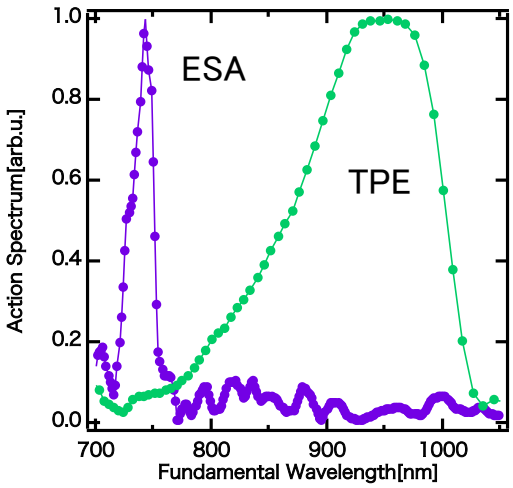

Fig.2 TPE and ESA spectra of eGFP.

4. Summary

In summary, the action spectrum for the photobleaching of a fluorescent protein was observed by using nonlinear Fourier-transform spectroscopy. We found that sequential three-photon excitation process, one-photon ESA following two-photon excitation from the ground state, is one of the routes to the photobleaching.

5. References

[1] W. R. Zipfel, R. M. Williams and W. W. Webb, "Nonlinear magic: multiphoton microscopy in the biosciences," *Nat. Biotec.* **21**, 1369-1377 (2003).

[2] H. Hashimoto, K. Isobe, A. Suda, F. Kannari, H. Kawano, H. Mizuno, A. Miyawaki, and K. Midorikawa, "Measurement of two-photon excitation spectra of fluorescent proteins with nonlinear Fourier-transform spectroscopy,"*Appl. Opt.* 49, 3323-3329 (2010).