Measurement of two-photon excitation spectrum used to photoconvert a fluorescent protein (Kaede) by nonlinear Fourier-transform spectroscopy

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Abstract: We demonstrate the measurement of two-photon excitation (TPE) spectra, used not only for fluorescence but also for photoconversion in green-to-red photoconvertible Kaede, using nonlinear Fourier-transform spectroscopy. It was found that in unphotoconverted Kaede, the TPE spectrum for photoconversion is much different to that for green-fluorescence. This is similar to the difference between the one-photon excitation of photoconversion in the neutral form and that of green-fluorescence in the ionized form.

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1. Introduction

The capability of two-photon fluorescence microscopy [1,2] in biological research has recently been extended by ultrafast laser technology [3,4] and by fluorescent probe technology [5–9]. The spectral phase modulation technique with a broadband laser pulse used to control two-photon excitation (TPE) processes [3,4] provides rapid and easy switching between multi-color imaging [10] and fluorescence resonance energy transfer imaging [11] for visualizing the dynamics of biomolecules and their interactions with cellular components in a living cell. TPE of photoactivated or photoconverted fluorescence proteins exhibiting photoinduced spectral changes [8,9] enables us to label a single organelle in a living cell and to track it [12,13]. TPE spectra of fluorescent molecules provide indispensable information for the use of these techniques. TPE spectra of the fluorescence [14–21] from various fluorophores have been measured by various spectroscopic techniques including the wavelength scanning method [14–16], the multiplex method [17] and the Fourier-transform (FT) method [18–21]. However, there are few reports on the measurement of TPE spectra for photoactivation [22] or photoconversion because of the difficulty in measuring them. In the multiplex method, since absorption is directly measured, it is hard to determine whether the absorption spectral change is induced by the excitation of photoconversion or of fluorescence. Thus, it is difficult to apply the multiplex method to the measurement of TPE spectra for photoconversion. In the case of the measurement of the TPE spectrum for photoconversion using the wavelength scanning method or the FT method, after irradiation by the pump light for photoconversion, it is necessary to record the photoinduced spectral change with a probe light. However, in the wavelength scanning method, it is difficult to precisely evaluate the amount of photoinduced spectral change because fluctuations from tuning the pump wavelength could affect the spatial overlap between the pump and probe lights. On the other hand, the FT method using broadband pulses allows us to measure the TPE spectrum at once without tuning the pump wavelength and to suppress the fluctuations in the spatial overlap between the two lights. In addition, the resultant spectrum is not significantly influenced by temporal fluctuations in the detected signal. However, the FT method has not been applied to the measurement of TPE spectra for photoactivation or photoconversion. In this article, we demonstrate the measurement of the TPE spectrum for photoconversion in Kaede using the FT method. Kaede is a photoconvertible fluorescent protein and has a green chromophore that can be converted to red [8]. We also show the TPE spectra for green- and red-fluorescence in unphotoconverted Kaede and photoconverted Kaede, respectively.

2. Materials and methods

2.1 Experimental setup

The experimental setup consists of two parts: a pump system for TPE photoconversion and a probe system for the photoinduced spectral change (See Fig. 1). The pump system, which is the same as that used for nonlinear FT spectroscopy for the measurement of the TPE spectra of fluorescent proteins [19–21], was used to induce green-to-red photoconversion in Kaede with TPE. The pump light source used for TPE photoconversion was a mode-locked broadband Ti:sapphire laser (Nanolayers, Venteon OS) operating at a repetition rate of 80 MHz. The laser spectrum ranged from 670 nm to 1150 nm. To generate a Fourier-transform-limited pulse at the sample, we pre-compensated for dispersion of the optical components by the use of a dispersion pre-compensator consisting of a fused-silica prism pair and a 4-f pulse shaper with a liquid-crystal spatial light modulator. The broadband pulse was passed through a Michelson interferometer to generate two pulses with a variable delay time between them. These pulses were then focused onto the sample with an objective lens (OB1) (Olympus Corp., UPLSAPO40 $\times$ NA = 0.9). A pulse duration at the sample of 5 fs was achieved by pre-compensation. TPE photoconversion was induced by the broadband pump light with a total average power of 60 μ W and a beam spot diameter of 1.6 μ m ($1/e^2$) at the sample. The probe system was used to quantify the magnitude of the induced photoconversion. We selected a continuous-wave laser (NTT Electronics, Opti λ) at a wavelength of 589 nm for the probe light source. The probability of generating green-fluorescence or photoconversion in Kaede with excitation at 589 nm is almost zero, while the 589 nm light induces red-fluorescence in photoconverted Kaede [8]. The probe light was collinearly combined with the broadband pump light using a dichroic mirror (DM1) (Semrock, FF662-FDi01-25 $\times$ 36) and was focused onto the sample. The power and beam spot diameter ($1/e^2$) of the probe light at the sample were 1.8 μ W and 1.2 μ m, respectively. Red-fluorescence was detected in the backward direction with a photomultiplier tube (PMT2) (Hamamatsu Photonics, H7422A-40) through a band-pass filter (BPF2) (Semrock, FF01-624/40-25) and a confocal pinhole (PH) with a diameter of 75 μ m. The TPE fluorescence intensity with the pinhole was reduced by nearly 30% from that without it. Since red-fluorescence could be generated not only by the probe light but also by the pump light, only the probe light was modulated by an optical chopper at a frequency of 3.97 kHz, and a red-fluorescence signal at that frequency was obtained through a lock-in amplifier (Stanford Research Systems, SR830).

Fig. 1. Experimental setup. PD: photodiode, BS: beam splitter, OB: objective lens, DM: dichroic mirror, PMT: photomultiplier tube, SPF: short-pass filter, BPF: band-pass filter, PH: pinhole.

2.2 Measurement of TPE spectrum for photoconversion

The interferometric autocorrelation (IAC) signal from TPE photoconversion was measured by scanning a piezoelectric-driven delay stage with a time-delay step of 98 as. The maximum delay time was set to be 200 fs. The IAC signal for each delay time was acquired using the following procedure. The exposure time of the pump light was controlled by an electromagnetic shutter (Sigma Koki, Σ -65L), which was opened and closed while the sample was constantly being irradiated with the probe light. The red-fluorescence signal was recorded before, during and after exposure to the pump light. After recording these, the focal point was moved to a region of the sample not exposed to pump light, by moving the sample which was mounted on a stepping motor. To determine the time the shutter was open in the obtained data, higher-order diffracted pump light from the grating in the pulse shaper was measured by a silicon photodiode (PD). We performed a linear least-squares fit of the time response of the red fluorescence signal ranging from when the shutter was opened till 200 ms after the shutter was opened. The slope of the fitted line, which indicates the number of photoconverted molecules per second, was used for the photoconversion signal for each delay time.

The TPE action spectrum $\sigma_s^{(2)}(\omega)\phi_s^{(PC)}$ for photoconversion was acquired using

$$\sigma_s^{(2)}(\omega)\phi_s^{(PC)} = \frac{1}{k} \frac{C_r \phi_r^{(f)} \alpha_r S_{PC}^{(2)}(\omega)}{C_s \phi_s^{(f)} \alpha_s S_{SH}^{(2)}(\omega)} \frac{1}{p(\omega_0) \sigma_s^{(1)}(\omega_0)}. \quad (1)$$

where $S_{SH}^{(2)}(\omega)$, $S_{PC}^{(2)}(\omega)$ and $\phi_s^{(PC)}$ are the reference second-harmonic (SH) spectrum, the Fourier spectrum of the IAC signal from TPE photoconversion and the photoconversion quantum efficiency, respectively. The SH spectrum was obtained by Fourier-transformation of the second-order IAC signal from second-harmonic-generation in the region near the surface of quartz in a non-phase matched configuration [21]. k is the calibration constant for the absolute spectrum and was acquired from $k = S_r^{(2)}(\omega) / S_{SH}^{(2)}(\omega) \sigma_r^{(2)}(\omega)$, where $S_r^{(2)}(\omega)$ was obtained by Fourier-transformation of the second-order IAC signal from Rhodamine B (700 μ M) whose absolute TPE spectrum $\sigma_r^{(2)}(\omega)$ is well known [16]. C_s and C_r are the concentrations, $\phi_s^{(f)}$ and $\phi_r^{(f)}$ are the fluorescence quantum yields, α_s and α_r are the fluorescence detection efficiencies for Kaede and Rhodamine B, respectively. $p(\omega_0)$ is the photon flux density at 589 nm and $\sigma_s^{(1)}(\omega_0)$ is the one-photon absorption cross section at 589 nm. In nonlinear FT spectroscopy for the measurement of the TPE spectrum for fluorescence, the measured SH spectrum is also used for the compensation of the chromatic aberration and the difference in spot size of the pump beam as a function of the wavelength. By equalizing the spot sizes of the pump and probe beams, the compensation by the SH spectrum is also available at the measurement of TPE spectrum for photoconversion using the probe beam.

2.3 Sample preparation

In order to suppress Brownian motion during the measurement at each delay time, Kaede in a viscous solution was prepared for the sample. A solution of Kaede in HEPES NaOH buffer (50 mM, pH 7.4) was diluted in glycerine to achieve a protein concentration of 47 μ M in a 75% glycerine-buffer mixture. Although we need to measure the concentration of the active protein that can be photoconverted, there is no method for investigating whether the protein can be photoconverted or cannot be photoconverted. Thus, the concentration was estimated from its molar absorption coefficient of 98,800 $M^{-1}cm^{-1}$ at 508 nm. Our experiment has the concentration error based on the ratio of the active protein. This solution was set in a glass container with an optical path length of 70 μ m and this was used for the sample. The diffusion coefficient of the protein in the 75% glycerine-buffer mixture was calculated to be 0.54 $\mu m^2/s$ from the viscosity of 36 cPoise, temperature of 20 $^{\circ}C$ and Stokes radius of 11 nm. In this calculation, we assumed from the diffusion coefficient given in Ref [23] that the Stokes radius of Kaede is nearly four times as large as that of a green fluorescent protein (GFP) [24]. From

this diffusion coefficient, the mean displacement for Brownian motion of the protein during an exposure time of 200 ms was estimated to be smaller than the focal spot sizes of the two excitation beams. Thus, we assumed that few proteins moved in or out of the focal spot and the measurement was not affected by Brownian motion.

To measure the TPE spectra for green-fluorescence in unphotoconverted Kaede and red-fluorescence in photoconverted Kaede with nonlinear FT spectroscopy [19], a solution of Kaede in HEPES NaOH buffer was set in two glass containers. One was used for the green-fluorescence measurement without preprocessing. The other was irradiated by excitation light at 405 nm to convert it from green- to red-fluorescence. The concentrations of the unphotoconverted (green) Kaede and photoconverted (red) Kaede solutions were estimated to be 78 μM and 61 μM from their molar absorption coefficients of 98,800 $\text{M}^{-1}\text{cm}^{-1}$ at 508 nm and 60,400 $\text{M}^{-1}\text{cm}^{-1}$ at 572 nm, respectively. The dwell time for each delay time was 200 μs . The diffusion coefficient of the protein in the buffer solution was calculated to be 20 $\mu\text{m}^2/\text{s}$. Since the dwell time was short enough and many proteins moved in and out of the focal spot due to the large diffusion coefficient, we ignored the effect of photoconversion in the green fluorescence measurement.

3. Results and discussion

We first obtained the red-fluorescence signal from photoconverted Kaede before, during and after irradiation by the pump light as shown in Fig. 2(a). The red solid line and the black dotted line indicate the photoconverted red fluorescence intensity and the pump intensity, respectively. After the shutter was opened, the fluorescence intensity increases because of the rise in the number of photoconverted proteins with increasing exposure time. After this the fluorescence intensity saturates due to the lack of unphotoconverted proteins in the focal spot. The fluorescence intensity has not yet reached saturation in the first 200 ms after opening the shutter. After closing the shutter, the fluorescence intensity is maintained for 200 ms. This result suggests the validity of the assumption that Brownian motion has no effect on the measurement. We measured the dependence of the photoconversion signal on pump power. The measured result of the dependence is shown in Fig. 2(b). The photoconversion signal is proportional to the square of the pump power. From this dependency, we can confirm that photoconversion was induced by TPE. Figure 2(c) shows the IAC signal from TPE photoconversion. We found that the fringe contrast is nearly 1:8. This fact also indicates that photoconversion was induced by TPE. Figure 3(a) shows the TPE action spectrum for photoconversion. The TPE action spectrum was obtained by averaging 10 times. Note that the fundamental wavelength shown on the upper horizontal axis for convenience is just twice the SH wavelength shown on the lower horizontal axis. We found that the two-photon excitation peak for photoconversion was located at around 390 nm.

Fig. 2. (a) Time responses of photoconverted red fluorescence signal (red solid line) and pump light (black dotted line). (b) Power dependence of photoconverted fluorescence signal. (c) IAC signal from photoconverted fluorescence.

We also measured the TPE spectra for green-fluorescence in unphotoconverted Kaede and red-fluorescence in photoconverted Kaede with nonlinear FT spectroscopy. Figures 3(b) and 3(c) show the absolute TPE spectra for green- and red-fluorescence, respectively. The TPE spectra for photoconversion and green fluorescence in Kaede are very similar to those for fluorescence in Sapphire and enhanced GFP (EGFP), respectively [20,21], which are GFP variants. The green state of Kaede shows two one-photon absorption peaks at 380 and 508 nm, corresponding to neutral and ionized forms, respectively [8]. Photoconversion is induced by excitation of the neutral form, whereas green-fluorescence is generated by excitation of the ionized form. This is analogous to the photocharacterization of GFP with one-photon excitation. Sapphire molecules contain neutral chromophores with a one-photon excitation peak at 399 nm, whereas EGFP molecules have ionic chromophores with a one-photon excitation peak at 488 nm [6]. From the photocharacterization of Kaede and GFP with one-photon excitation and the similarity of the TPE spectra in Kaede and in GFP, it appears that photoconversion and green-fluorescence in Kaede on TPE are also induced by the excitation of the neutral and ionized forms, respectively.

As for the photoconverted Kaede, the TPE spectrum for red-fluorescence from Kaede is similar to that from DsRed [20,21]. Fluorescence from DsRed with TPE at 750 nm bleaches more rapidly than that at 950 nm owing to three-photon absorption at 750 nm [25]. Although

we have not compared the photobleaching speed at a short wavelength (< 800 nm) with that at a long wavelength (> 900 nm), we hypothesize that red-fluorescence from Kaede with TPE at a short wavelength (< 800 nm) might bleach readily, like the photobleaching in DsRed because of the similarity of the TPE spectra in DsRed and in red-Kaede. If this hypothesis is correct, photoconversion in Kaede would tend to be followed by photobleaching because of the same effective excitation wavelength for red-fluorescence photobleaching as that for photoconversion. Nonlinear FT spectroscopy can be applied to investigate photobleaching. In the future, we will verify this hypothesis by nonlinear FT spectroscopy.

Fig. 3. TPE spectra for (a) green-to-red photoconversion, (b) green fluorescence (c) red fluorescence.

In nonlinear FT spectroscopy using a Fourier-transform-limited (FTL) pulse, the signal-to-noise ratio (SNR) is low at the both ends of the SH wavelength range because the SH intensities at the ends is much lower than that at center SH wavelength. The SNR at the ends can be improved by shaping the SH spectrum to be nearly flat and by increasing the average power of the pump pulse. The SH spectrum can be easily shaped by modulating the spectral phase of the pump pulse [3,4,20].

We have demonstrated the application of nonlinear FT spectroscopy for the measurement of the absolute TPE spectra not only for fluorescence but also for photoconversion in a photoconvertible fluorescent protein (Kaede). This can be a useful technique for developing novel functional fluorescent proteins and for optimizing TPE microscopy.

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