Nonlinear fluorescence imaging by photoinduced charge separation

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Manipulation of the optical property of fluorescent probes has been a powerful strategy to establish super-resolution microscopy. We describe a new strategy to realize a probe with a nonlinear fluorescence response by using photoinduced charge separation. In this scheme, the first photon is used for the generation of the charge-separated state and the second photon is for fluorescence excitation. This stepwise two-photon absorption was confirmed by detection of a second-order nonlinear fluorescence response. Transient absorption spectra studies and simulation indicate that fluorescence is emitted through the photophysical pathways we proposed. Fluorescence imaging of biological cells showed marked improvements in image contrast and resolution, demonstrating the usefulness of the fluorescent probe in laser scanning confocal microscopy.

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1. Introduction

Optical microscopy enables the visualization of distributions and dynamics of intracellular structures and has been used as an inevitable tool for clarifying various cellular mechanisms. Although the spatial resolution of conventional optical microscopy has been limited by the diffraction limit, recent developments of super-resolution microscopy techniques have overcome this limit.1–5 A key to these developments of super-resolution microscopy is the manipulation of the emission properties of the fluorescent probe. Stimulated emission depletion microscopy and saturated excitation microscopy realize super-spatial resolution by utilizing the nonlinearity between excitation and fluorescence intensity caused by the saturation effect.6,7 The saturation effect has also been utilized in structured-illumination microscopy, and a spatial resolution of approximately 50 nm was demonstrated.8 In localization microscopy, a photoswitchable fluorescent protein/dye is used and has a 20–30 nm resolution for biological samples.9,10

In this report, we propose a fluorescent probe providing a nonlinear fluorescence response through intramolecular photoinduced electron transfer (PET) and subsequent charge separation. In the proposed scheme, one visible photon is used to switch a molecule to a switchable state, and another photon is used for fluorescence emission. The requirement of multiple photons for exciting a single fluorescence photon results in a nonlinear relationship between excitation and fluorescence intensity. As seen in two-photon microscopy, the nonlinear fluorescence response can be used to improve the spatial resolution of laser scanning microscopy. However, in typical two-photon microscopy, NIR light is used for excitation, and the resultant spatial resolution is similar to that of a single-photon excitation at visible wavelengths. On the other hand, the proposed fluorescent probe shows the nonlinear response under excitation at a visible wavelength; therefore, this probe can provide a marked improvement in spatial resolution of a typical confocal microscope.

2. Experimental methods

The proposed scheme is shown in Fig. 1. The molecular probe consists of two electron donors and one electron acceptor, where only the donor part is capable of exhibiting fluorescence and can absorb excitation light, and no interaction between the acceptor and the excitation light is assumed. When one of the two donors is excited by a single photon, a charge-separated state is formed through PET without emitting a fluorescence photon. Under this condition, the probe can still absorb another photon through the other donor and emit fluorescence, assuming that only one donor can form the charge-separated state with the acceptor. Since the fluorescence emission requires the interaction of the probe with two photons, the nonlinear relationship between excitation and fluorescence is expected. The fluorescence intensity is then no longer linearly proportional to the excitation light intensity, and consequently, the point-spread-function (PSF) of the fluorescence excitation becomes smaller than that in single-photon excitation. Therefore, the spatial resolution can be improved by using the proposed fluorescent probe in a conventional confocal microscope. A similar idea for inducing the nonlinear fluorescence response using fluorescence resonance energy transfer (FRET)11–14 and bright-dark state switching of dyes has been proposed.15 However, the approach using intramolecular PET has not yet been investigated.

Fig. 1. (Color online) Schematic of the donor–acceptor–donor structure and the proposed scheme of the nonlinear fluorescence emission through molecular switching by PET. D and A represent an electron donor and an electron acceptor, respectively. The symbols ‗+‘, ‗+‘, and ‗–‘ denote an excited state, a positive charge, and a negative charge, respectively.

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3. Results and discussion

3.1 Synthesis of fluorescence probe with charge separation

To confirm the proposed photophysical response, we synthesized a probe consisting of two boron-dipyromethene (BODIPY) dyes as electron donors and one nitrobenzene molecule as an acceptor as shown in Fig. 2(a), and named it nitro-bisBODIPY. BODIPY is a novel fluorescent dye with attractive features, such as high photostability and high fluorescence quantum yield in various solutions, which can be tuned by chemical modification.20–23 On one hand, nitrobenzene is known to quench the fluorescence of a fluorophore via intramolecular PET owing to its low LUMO energy level.20,21 In order to optimize the efficiency of electron transfer between BODIPY and nitrobenzene, they were placed orthogonally and bonded only with a single carbon bond. A molecule with a single donor and an acceptor was also synthesized [nitro-monoBODIPY, Fig. 2(b)] to confirm the efficiency of PET between the donor and the acceptor. A similar compound with a single BODIPY group was investigated previously and a fluorescence quantum yield of <0.01 resulting from the quenching of the fluorescence of BODIPY was obtained.21 We also synthesized a molecule where two BODIPY molecules are connected with a benzene ring, which does not act as an electron acceptor, in order to confirm the fluorescence response of two closely located BODIPY molecules [bisBODIPY, Fig. 2(c)]. Please see the online supplementary data at http://stacks.iop.org/JJAP/54/042403/mmedia for details of the synthesis.

3.2 Fluorescence response of nitro-bisBODIPY

We measured the fluorescence intensities of the synthesized molecules with various excitation intensities to confirm the nonlinear fluorescence response induced by molecular switching. A 488-nm CW laser (Coherent SAPHIRE 488-20 CDRH) was used as the excitation light source and focused into a sample solution using a 1.2 NA objective lens (Olympus UPlanApo 60x/1.20 W). The fluorescence from the solution was detected by an avalanche photodiode (Optoelectronics Photon Counting Module SPCM-AQR-16) through a confocal pinhole of 30 µm diameter equivalent to 0.5 airy units of the detection PSF. The concentration of the molecules in each sample solution was 10 µM in methanol. Dimethyl sulfoxide (DMSO) was used as a cosolvent for nitro-bisBODIPY and nitro-monoBODIPY, whereas chloroform was used for bisBODIPY. Figure 3 shows the fluorescence response obtained using the synthesized molecules. As we expected, nitro-bisBODIPY exhibited a second-order nonlinear fluorescence response to the excitation intensity. We also confirmed that nitro-monoBODIPY showed a weak and linear fluorescence emission, which indicates an efficient electron transfer induced between BODIPY and nitrobenzene. Nitro-bisBODIPY and nitro-monoBODIPY show similar fluorescence signal intensities at a low excitation intensity. However bisBODIPY showed a strong linear fluorescence emission at a high excitation intensity, which rules out the possibility that closely located two BODIPY molecules could contribute to the nonlinear fluorescence emission. It follows from the above results that the nonlinear fluorescence response of nitro-bisBODIPY is induced by PET between the donor and the acceptor of the molecule. The comparison of fluorescence signals from nitro-bisBODIPY and bisBODIPY at a low excitation intensity shows >99% suppression of the fluorescence emission from nitro-bisBODIPY by the electron transfer.

3.3 Theoretical investigation of fluorescence response of nitro-bisBODIPY

For the theoretical investigation of the nonlinear fluorescence response observed in the experiment, we built a model describing the energy transitions of nitro-bisBODIPY upon light excitation (Fig. 4). The energy diagram consists of five energy states that include a ground state, an excited state in which only one of the donors is excited by an incident photon, another excited state in which both donors are excited, a charge-separated state caused by PET between a donor and an acceptor, and a last excited state where a donor is excited by another photon incidence while the other donor and the acceptor form a charge-separated pair. The nonlinear fluorescence response can be realized by considering the energy transitions following the paths from (i) to (iv). (i) When one of the donors is excited by an incident photon, (ii) a charge-separated state is formed through intramolecular PET without fluorescence emission. (iii) The other donor
can then be excited by another incident photon under this charge-separated state, and (iv) the probe finally emits a fluorescence photon. Another possible pathway is through the simultaneous excitation of both donors (ii') and subsequent electron transfer to form a charge-separated pair and an excited donor (iii'). To determine the parameters to be used in the model, we measured the transient absorption spectrum of nitro-bisBODIPY, which can provide an estimate of the time constant of the electron transfer and lifetime of the charge-separated state. The transient absorption spectrum was measured by a pump–probe technique using an excitation pulse with a pulse width of 200 fs. The source for the pump and probe light were derived from a femtosecond pulsed laser (800 nm, 1 kHz). The 510-nm pulsed pump beam was generated by introducing the light source into an optical parametric amplifier. Figures 5(a) and 5(b) shows the transient absorption spectra of nitro-bisBODIPY and bisBODIPY measured in a 8000 ps time window after excitation by a femtosecond pulse, respectively. Two negative and two positive absorbance changes were observed at approximately 480 and 500 nm and 420 and 530 nm, respectively. The time traces of these changes after the excitation were analyzed by fitting with mono- or bi-exponential decay functions.

Since the wavelengths of 480 and 500 nm correspond to the absorption peaks of nitro-bisBODIPY, the bleaching and recovery of the ground state after excitation can be monitored from the absorbance change at these wavelengths. Figure 5(b) shows the average time trace of absorbance changes in the wavelengths of 490–500 nm. Immediately after the excitation by single-pulse light, the molecule starts losing the absorbance, approaching the maximum within 1 ps. This absorbance loss recovers with a lifetime of 13 ps, which indicates the presence of a short-lived excitation state in nitro-bisBODIPY.

The transient absorption spectra show a positive transient band at approximately 530 nm, which can be attributed to the charge-separated state. Hattori et al. reported that this relatively weak positive peak in this range is attributed to

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**Fig. 4.** (Color) Estimated five-state energy diagram of nitro-bisBODIPY. Ex, excitation; Em, fluorescence emission; IC, internal conversion; SE, stimulated emission; ET, electron transfer; ER, electron recombination.

**Fig. 5.** (Color) Transient absorption spectra of (a) nitro-bisBODIPY and (b) bisBODIPY in the range of 1–8000 ps after excitation by 510-nm femtosecond pulsed laser. Averaged time trace of absorbance changes at the wavelengths of 490–500 nm indicating the ground state of (c) nitro-bisBODIPY and (d) bisBODIPY. Averaged time trace of absorption changes at the wavelengths of 520–540 nm of (e) nitro-bisBODIPY, and (f) bisBODIPY.
the generation of a radical cation of the BODIPY moiety. The average time trace of absorbance change at the wavelengths of 520–540 nm was monitored, as shown in Fig. 5(c). Since the contribution of absorbance loss due to ground state bleaching still remains in this time scale, the time trace was fitted with bi-exponential decay functions with positive and negative coefficients. The extracted positive absorbance change after excitation is assumed to be due to the generation and decay of the charge-separated state because only the BODIPY dyes with a nitrobenzene molecule showed this transient band, but not bisBODIPY. The absorbance change at the wavelengths of 520–540 nm quickly increases soon after excitation, reaching the maximum within 1 ps, and then decaying with a lifetime of 12 ps. This result implies that the charge-separated state is generated within 1 ps after light excitation and has a lifetime of 12 ps.

Combined with the result from monitoring the ground state, the energy transitions of nitro-bisBODIPY after a single pulse excitation can be summarized as follows. After excitation, the energy state of nitro-bisBODIPY transits to the excited state (<1 ps). Then it changes to the charge-separated state within 1 ps via intramolecular PET. Finally, the molecule changes back to the ground state with a lifetime of 12 ps. It can be assumed that the nonlinear fluorescence response occurs when one more photon is absorbed by the molecule through the other donor within the 12 ps lifetime of the charge-separated state. The positive absorbance change at approximately 420 nm presumably indicates the generation and decay of the excited state because other dyes including BODIPY and bisBODIPY showed the same response. However, it was difficult to determine the transition rate because the wavelength region overlaps with the absorption peak of the radical anion of nitrobenzene.

On the basis of the five-energy-state model shown in Fig. 4, we constructed a series of rate equations describing the population of each state in the estimated five-energy-state model shown in Fig. 4 and solved it to estimate the fluorescence response of nitro-bisBODIPY. The response was obtained by calculating the sum of the populations of D'-A-D', D-A-D', and D'-A-D' states, which linearly determines the rate of all D to D' transitions, at various excitation intensities. The absorption/stimulated-emission cross section (1.7 × 10^{-16} cm²) and the rate constant of excited state decay (9.7 × 10^9 s⁻¹) used in the calculations were experimentally obtained from the absorption and transient absorption spectra.

For the rate constant of electron transfer, we examined several values on the basis of the results of the transient absorption measurement since the transient absorption spectra implied that the electron transfer occurs within a time shorter than 1 ps after the excitation. Figure 6(a) shows the simulation results for the fluorescence response with increasing excitation intensity at different electron transfer rate constants (k_{ET}). (0.01 ps)^{-1}, (0.1 ps)^{-1}, (0.2 ps)^{-1}, (0.3 ps)^{-1}, (0.4 ps)^{-1}, and (0.5 ps)^{-1} were used for k_{ET}, whereas the rate constant of electron recombination (k_{ER}) was fixed to (12 ps)^{-1}, which was measured in the experiment. At a small k_{ET}, the quantum efficiency of PET is lower than at a large k_{ET}, resulting in the enhancement of the linear fluorescence response. To compare the simulation results with the experimental results, we focus on the blue square region in Fig. 6(a), which corresponds to the actual excitation intensities used in the experiment. In this region, the simulated fluorescence response, where k_{ET} is (0.2 ps)^{-1}, exhibits a second-order nonlinear response that matches well with the experimental optical response of nitro-bisBODIPY. Therefore, a good estimate of the rate constant of electron transfer in nitro-bisBODIPY is (0.2 ps)^{-1}. As described above, a window of 12 ps (derived from the lifetime of the charge-separated state) is enough to allow nitro-bisBODIPY to absorb a second photon after absorbing the first photon for realizing nonlinear fluorescence emission. The number of photons incident on the focal spot within 12 ps is estimated to be 5,600 at an excitation intensity of 10^8 W/cm², at which the nonlinear response starts to appear.
in Fig. 3. Hence, nitro-bisBODIPY requires, at the least, an excitation by a visible CW source while a typical two-photon absorption molecule needs an ultrashort pulse light source to realize simultaneous two-photon absorption on the femtosecond order.36)

To gain more insights about our nonlinear fluorescent probe, we calculated the fluorescence response of nitro-bisBODIPY at various values of $k_{\text{ER}}$ [Fig. 6(b)]. The calculation result indicates that the second-order nonlinear response appears with a relatively small $k_{\text{ER}}$. At a large $k_{\text{ER}}$, the charge-separated state seems to dissolve before absorption of the second excitation photon. As seen in the plot with $k_{\text{ER}} = (1.2 \text{ ps})^{-1}$, the quadratic response appears at a higher excitation intensity at which the absorption of the second photon can occur before the molecule can go back to its initial state. At a small $k_{\text{ER}}$, it is obvious that the nonlinear response can be observed even at a low excitation intensity. The chemical modification of nitro-bisBODIPY or the selection of a different type of electron donor–acceptor pair to increase the lifetime of the charge-separated state may result in the nonlinear fluorescence response with a much lower excitation intensity. At the same time, however, the fluorescence intensity easily saturates because two or more photon incidences on the molecule is allowed before the relaxation to the initial state, where three or four excitations are used to obtain the response.

### 3.4 Fluorescence imaging with nitro-bisBODIPY

Finally, we performed fluorescence imaging of fixed HeLa cells using nitro-bisBODIPY and investigated the spatial resolution improvement. HeLa cells were immersed in 10 µM dye solution diluted with methanol at room temperature after washing twice with phosphate-buffered saline (−). In this process, the cells were stained and fixed simultaneously. After immersion for 30 min, the cells were washed twice, and then finally immersed in methanol. The fluorescence images of cells were measured with a confocal microscope with a pinhole of 0.7 airy unit size, and excitation was provided by a 488-nm CW laser. A 1.2 NA water-immersion objective lens was used for the observation. Figure 7 shows the XY and XZ fluorescence images of HeLa cells stained with nitro-bisBODIPY and BODIPY. The two probes have similar fluorescence spectra at 500–600 nm with a 4 nm difference in the peak, and the wavelength range of fluorescence detection was 500–900 nm. There was no significant problem in using the two images for the comparison of image contrast and resolution. Since two different samples were compared, it is difficult to confirm the resolution improvement clearly. However, comparison of the image contrast in the areas of the cell containing dense droplets showed the marked improvement in the quality of the image when using nitro-bisBODIPY, presumably as a result of the contrast enhancement or resolution improvement due to the nonlinear fluorescence response of the probe. We also obtained fluorescence images of HeLa cells stained with bisBODIPY, and it was confirmed that the distribution of the probes in cells was similar to that of BODIPY, indicating that nitro-bisBODIPY and BODIPY distributed in the same cellular organelles.

### 4. Conclusions

We have designed, synthesized, and demonstrated a novel fluorescent probe called nitro-bisBODIPY exhibiting a nonlinear fluorescence response mediated by the photoinduced electron transfer effect. The energy transitions of nitro-bisBODIPY that account for the nonlinear fluorescence response were examined theoretically by measuring transient absorption spectra and simulation of the optical response. The practical utility of the probe for high-resolution microscopy was demonstrated by cellular fluorescence imaging. With the compound we developed, the excitation intensity required to induce nonlinear fluorescence emission is much lower than that required for a typical two-photon excitation achieved by an ultrashort pulsed laser. Because repetitive single photon excitation is used, even a CW laser can produce the nonlinear fluorescence emission. For the same reason, the wavelength of excitation light can be shorter than that used for typical two-photon excitation, which allows us to maintain the spatial resolution in laser scanning microscopy. The spatial resolution achieved by using DUV light can also be accomplished by using visible light and ordinary optical components. This nonlinear property of the molecules can also be useful for increasing the resolution of photolithography and density of data storage.

Although we have obtained the nonlinear response via charge separation, the quantum yield of the emission of nitro-bisBODIPY seems small, which was difficult to obtain from our experiment, but can be estimated from that of bisBODIPY (~0.02). The use of different combinations of fluorophores needs to be investigated to increase the fluorescence signal intensity in imaging. It is also expected for the PET scheme that the order of nonlinearity can be tuned by changing the number of charge-separated states formed in a single molecule.

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