High-resolution fluorescence microscopy based on a cyclic sequential multiphoton process

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Abstract: We demonstrate high-resolution fluorescence microscopy based on a cyclic sequential multiphoton (CSM) process, which gives rise to fluorescence emission following a sequence of cyclic transitions between the bright and dark states of a fluorophore induced by pump and reverse light. By temporally modulating the reverse intensity, we can extract the fluorescence signal generated through the CSM process. We show that the demodulated fluorescence signal is nonlinearly proportional to the excitation intensities and it gives a higher spatial resolution than that of a confocal microscope.

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1. Introduction

Confocal fluorescence microscopy is a versatile technique for three-dimensionally visualizing biological phenomena such as the expression of genes, ionic concentration changes, and membrane potential changes [1–6]. However, confocal fluorescence microscopy is not used to investigate biological phenomena in intracellular organelles smaller than 200 nm in size owing to the limit set on the spatial resolution by the diffraction of light. To further explore biological phenomena, two distinct microscopic techniques have been attempted to break the diffraction limit [6–23] and have already been applied to live-cell imaging [20–23]. One technique uses saturation phenomena in the optical effects, including stimulated emission depletion (STED) microscopy [7], saturated structured-illumination microscopy (SSIM) [8], saturated excitation (SAX) microscopy [9]. To induce saturation phenomena, high-intensity excitation, which can cause sample damage, is typically required. Although STED microscopy with continuous wave (CW) laser beams has been reported [19], a picosecond pulse laser, which is expensive, is typically used for STED microscopy. In addition, STED microscopy requires the temporal and spatial adjustment of two laser pulses from different light sources, which significantly complicates the construction of the system. The second technique for super-resolution imaging is based on the single-molecule localization [10–18]. Localization microscopy has managed to reconstitute full images from many thousands of localized molecules, where the localization error of single molecules can be far smaller than the optical resolution. Localization microscopy using photoswitchable fluorescent molecules was developed by some groups and was termed photoactivated localization microscopy (PALM) [13], fluorescence photoactivation localization microscopy (FPALM) [14], stochastic optical reconstruction microscopy (STORM) [15], or PALM with independently running acquisition (PALMIRA) [16]. Localization microscopy was also performed by utilizing fluorescence lifetime of conventional fluorescent dyes [17] or reversible photobleaching of conventional fluorescent proteins [18]. Localization microscopy usually requires a highly sensitive multi-element detector, which is expensive. An analysis of the acquired data is also needed for localization microscopy.

In this article, we propose a cyclic sequential multiphoton (CSM) excitation technique that makes high-resolution fluorescence microscopy less expensive and more accessible to

laboratories. Our technique is based on a sequential multiphoton process, which induces fluorescence emission following a sequence of cyclic transitions between two energy states in a single molecule. The nonlinearity of the sequential multiphoton process, which is similar to that of multiphoton excitation fluorescence [24,25], can be increased by increasing the cycle numbers of cyclic transitions. The nonlinearity breaks the diffraction limit. We demonstrate the application of the CSM excitation technique to improve the spatial resolution of a laser scanning fluorescence microscope.

2. Principle of cyclic sequential multiphoton microscopy

Here, we describe the principle of CSM microscopy. Let us assume a fluorophore with the photocycle shown in Fig. 1. With excitation by pump light at λ_p , the fluorophore is converted from a fluorescent state A to a non-fluorescent state B via an excited state A^* , and with excitation by reverse light at λ_r , it is backconverted from B to A via a different excited state B^* . The excitation by the pump light also induces fluorescence emission from the fluorophore with a certain probability. If the lifetimes of A^* and B^* are much shorter than the observation time scale, we can regard the fluorescence emission following a sequence of cyclic transitions between A-to-B and B-to-A in the single fluorophore as a multiphoton process. For example, the cyclic transitions are caused by alternating between absorption and stimulated emission or by repeating reversible photoswitching. In a cyclic sequential $2n + 1$ photon process, fluorescence is emitted at the final stage after the cycles of transitions between A-to-B and B-to-A have been repeated n times. The fluorescence intensity in a n -cycle sequential $2n + 1$ photon process is proportional to $I_p^{n+1} I_r^n$, where I_p and I_r are the excitation intensity of the pump and reverse light, respectively. In conventional one-photon fluorescence microscopy, the fluorescence intensity is proportional to the excitation intensity. On the other hand, the fluorescence intensity in conventional n th photon fluorescence microscopy is proportional to the n th power of the excitation intensity. Because of the nonlinearity, the diffraction limit is broken [24,25]. However, the spatial resolution is $\sqrt{n}$ times lower than that of one-photon fluorescence microscopy owing to the excitation wavelength being n times longer than that of one-photon excitation. The nonlinearity of the fluorescence in the n -cycle sequential $2n + 1$ photon process, which is similar to that of multiphoton excitation fluorescence [24,25], can be used to improve the spatial resolution by a factor of $\sqrt{2n+1}$, which is beyond the diffraction limit, due to the excitation being the same wavelength as one-photon excitation. Moreover, the spatial resolution of a CSM microscope is not limited under unsaturated conditions. However, when at least one linear optical phenomenon in the CSM process is saturated, the nonlinearity is decreased. Thus, the excitation intensity levels must be controlled in unsaturated conditions.

Fig. 1. Schematic diagram of a cyclic sequential multiphoton process.

To achieve CSM microscopy, an important problem must be solved. In the focal volume, there are the fluorophores which transit at a variety of cycle numbers before fluorescence emission. In addition, fluorescence is also generated from fluorophores which do not transit between the two states. Thus, to identify the order of a CSM process, we must recognize through which process the fluorescence signal is emitted. To solve this problem, we

temporally modulate the excitation intensity of the reverse light at a frequency of ω and determine the cycle number in the CSM process. By expressing the reverse light intensity as $I_r = A_r\{1 + \cos(\omega t)\}$, the fluorescence intensity for n -cycle CSM excitation can be described by $I_f \propto I_p^{n+1} A_r^n \{1 + \cos(\omega t)\}^n$. Then, the demodulated fluorescence intensity at a frequency of $n\omega$ ($n = 0, 1, 2, \dots$) is mainly proportional to $I_p^{n+1} A_r^n \cos(n\omega t)$. Therefore, we can acquire the $2n + 1$ th order nonlinear signal by detecting the frequency component at $n\omega$. The excitation intensity of the CSM microscope is much lower than the multiphoton microscope and super-resolution microscope employing the saturation effect because of the unsaturated excitation of the linear optical process. Thus, CSM microscopy can be achieved by low-power laser diode (LD) systems which provide a low cost.

3. Experimental

We experimentally confirmed the principle of CSM microscopy by the use of Dronpa-3, which is a reversible photoswitchable fluorescent protein [26]. With excitation at a wavelength of 488 nm, Dronpa-3 normally emits fluorescence and can be rarely converted from a fluorescent to a non-fluorescent state. The protein in the non-fluorescent state can be switched back to the fluorescent state with irradiation at a wavelength of 405 nm. For the optical setup, we used a confocal fluorescence microscope with a single mode fiber (SMF) for delivering the excitation light from two LDs (Neoark, TC20-4820-4.5 and TC20-4030-4.5) which operate at wavelengths of 488 nm (pump light) and 405 nm (reverse light). The two laser beams were superimposed by using a dichroic mirror (DM; Semrock, Di01-R405) and were coupled into the SMF (Nufern, S405) with a mode field diameter of 2.9 μm at a wavelength of 405 nm. Modulation of the reverse laser intensity (405 nm) was performed by injection current modulation of the LD at a frequency of 400 Hz. Output beams from the SMF were focused into a sample by an objective lens with an effective numerical aperture (NA) of 0.7. Fluorescence from the sample was detected by a photomultiplier tube (PMT; Hamamatsu Photonics, R4220) through a DM (Semrock, FF502-670-Di01), a band-pass filter (Semrock, FF01-520/35), and a confocal pinhole with a diameter of 40 μm .

To confirm that the nonlinearity of the signal was induced by the CSM excitation, the demodulated fluorescence intensity was measured with various excitation intensities. Then, the ratio of the reverse power to the pump power was fixed to be 100. Figure 2(a) shows the Fourier components of the fluorescence signal from a Dronpa-3 solution with a concentration of 64 μM . We found that the single cycle component (ω) appeared in the fluorescence signal. Although multi-cycle components should also appear, they are probably buried in the noise due to the low signal-to-noise ratio (SNR). By increasing the SNR, multi-cycle components could be detected. Figure 2(b) shows the dependence of the fluorescence intensities at frequencies of 0 and ω on the total excitation intensity. At a low excitation intensity level ($< 500 \text{ W/cm}^2$), the fluorescence intensity at a frequency of ω is proportional to the 2.5th power of the total excitation intensity, whereas that at a frequency of 0 is almost proportional to the total excitation intensity. This result indicates that the nonlinearity of the CSM process can be applied to improve the spatial resolution. However, the fluorescence intensity at a frequency of ω should be proportional to the 3rd power of the total excitation intensity because the ω component represents the signal induced through the 1-cycle 3-photon process. It appears that the nonlinearity was reduced by saturation in the photoswitching and/or a transition to unknown dark states.

Fig. 2. (a) Fourier components of the fluorescence signal from a Dronpa-3 solution with a concentration of 64 μM . The modulation frequency was 400 Hz. (b) Dependence of the fluorescence intensity at frequencies of 0 and ω on the total excitation intensity.

To extract the frequency components at ω from the fluorescence signal, the fluorescence intensity was obtained through a lock-in amplifier (Stanford Research Systems, SR830). Figure 3 shows the lock-in signals from the Dronpa-3 solution. As shown in Fig. 3, the lock-in signal obtained by simultaneous excitation at both 488 nm (3.5 μW , 340 W/cm^2) and 405 nm (35 nW, 4.1 W/cm^2) is large, while those by excitation at only 488 nm (3.5 μW , 340 W/cm^2) or 405 nm (35 nW, 4.1 W/cm^2) are almost 0. This result shows that the fluorescence signal at a frequency of ω is given by a combination of 488 nm and 405 nm and not by the fluorescence originating from absorption at 405 nm. Thus, this confirms that the lock-in signal at a frequency of ω is induced by sequential multiphoton excitation.

Fig. 3. Lock-in signals obtained with excitation at only 488 nm (3.5 μW), at only 405 nm (35 nW) and at both 488 nm (3.5 μW) and 405 nm (35 nW).

The lateral and axial responses of the CSM and confocal microscopes were observed in order to demonstrate the improvement in spatial resolution. By employing Dronpa-3 immobilized on the surface of the amino-modified glass slide (Matsunami Glass, MAS-GP) as a sample, we measured one-dimensional (1D) signal intensity distributions along the lateral and axial directions near the interface between the glass slide and the protein. Then, the sample was scanned by utilizing a three-axis piezoelectric transducer (PZT) stage (Piezosystem Jena, TRITOR 101 CAP). The lock-in amplifier was used in CSM microscopy, while it was not used in confocal microscopy. In the axial direction, the fluorescent surface layer was so thin that the axial resolution could be directly obtained by the full width at half-maximum (FWHM) of the signal distribution. However, in the lateral direction, the spatial resolution was indirectly estimated from the edge response for glass/protein layer. Figures 4(a) and 4(b) show the 1D signal intensity distributions along the lateral and axial directions. In Fig. 4(a), the response of the CSM microscope is steeper than of the confocal microscope. By differentiating the 1D intensity distributions along the lateral direction, the lateral resolutions of the CSM and confocal microscopes were estimated to be 310 nm and 570 nm, respectively. The lateral resolution of the CSM microscope was almost twice as high

as that of the confocal microscope. As shown in Fig. 4(b), the axial resolutions of the CSM and confocal microscopes were $0.48\ \mu\text{m}$ and $1.48\ \mu\text{m}$, respectively. The axial resolution of the CSM microscope was almost 3 times higher than that of the confocal microscope. The improvement in spatial resolution in three-dimensions was achieved by the use of the CSM excitation. We consider that this improvement was assisted by using reverse light at a wavelength shorter than that of the pump light, because the diffraction-limited spot-size depends on the wavelength. Because the improvement rate of the spatial resolution does not depend on the numerical aperture, we could achieve a spatial resolution beyond the usual 200 - 250 nm laterally and 500 - 700 nm axially by using an objective lens with a numerical aperture of 1.2 or 1.4.

Fig. 4. 1D signal intensity distributions along the (a) lateral and (b) axial directions near the interface between the glass slide and Dronpa-3. Dronpa-3 was immobilized on the surface of MAS-coated glass slide.

Finally, we demonstrate fluorescence imaging of a Dronpa3-coated sphere. By employing the avidin-biotin interaction, avidin-microspheres (Micromod, sicastar) with a diameter of $3\ \mu\text{m}$ were labeled with biotinylated Dronpa-3. The Dronpa-3-coated spheres were spin-coated on the surface on a glass slide. Figure 5(a) and 5(b) show fluorescence images of the sample obtained by CSM and confocal microscopy, respectively. Figure 5 also illustrates 1D intensity profiles along the dotted lines shown in the fluorescence images. The total excitation powers in CSM and confocal microscopy were $1\ \mu\text{W}$ and $0.5\ \mu\text{W}$ corresponding to excitation intensities of $98\ \text{W}/\text{cm}^2$ and $48\ \text{W}/\text{cm}^2$, respectively. The pixel dwell time for both images was 10 ms. We found strong signals in the central region of the sphere in the confocal image as shown in Fig. 5(b). On the other hand, in the CSM image shown in Fig. 5(a), the signals in the central regions of the spheres are weak. Because Dronpa-3 was only coated on the surface of the sphere, fluorescence should not be generated from the central region. The contrast ratios ($=$ surface signal / center signal) in CSM and confocal images were 5.01 and 1.67, respectively. The reason for the difference in the fluorescence signal from the central region is because the axial resolution of the confocal microscope is equal to the bead radius, while that of CSM microscope was 3 times smaller than the bead radius. This result also indicates the improvement in spatial resolution.

Fig. 5. Fluorescence images of a Dronpa-3-coated sphere obtained by (a) CSM and (b) confocal microscopy, and intensity profiles along the green dotted lines shown in the images. Only the surface of the $3\ \mu\text{m}$ diameter sphere was coated with Dronpa-3.

4. Discussion and conclusion

We described the use of CSM excitation in confocal fluorescence microscopy in order to improve the spatial resolution in three-dimensions. Although the spatial resolution of CSM

microscopy is not limited theoretically, it is experimentally limited by the SNR and the dynamic-range of the detection system, the optical properties of the fluorophores, and the nonlinearity caused by the irradiation and detection systems. Therefore, in the future, the spatial resolution could be further improved by developing a detection system with a high SNR and a wide dynamic range, and fluorophores with high photoswitching quantum efficiency and low fluorescence quantum efficiency.

The CSM excitation could be also achieved by using conventional fluorescent molecules. In this case, instead of the cyclic transitions based on photoswitching, we could utilize the cyclic transitions between the excitation and ground states by the excitation with the pump light and by the stimulated emission with the reverse light.

The CSM excitation technique could be combined with localization microscopy such as PALM, FPALM PALMIRA and STORM. Both CSM microscopy and localization microscopy use the fluorescence emission process following the photoswitching that is induced by the excitation light with the sufficiently weak excitation intensity. The illumination and detection scheme in CSM microscopy is different from that in localization microscopy. Localization microscopy typically employs the wide-field illumination and detection scheme. On the other hand, CSM microscopy requires the focused illumination technique such as conventional nonlinear microscopy. This is because the spatial resolution can be improved by combining the nonlinearity in CSM excitation with the use of the tight focusing beam. Thus, the illumination and detection scheme needs to be modified. By using the stochastic scanning multifocal illumination [27] and wide-field detection scheme, the CSM technique could be applied to localization microscopy. This approach would be a nonlinear variant of localization microscopy and localization microscopy could be given the optical sectioning capability of nonlinear microscopes.

The CSM excitation technique described here can also be applied to various multicolor microscopies employing the sequential excitation of two processes in a single molecule. The first process is a cyclic transition process between two states. The excitation of the second process is used to generate a signal for imaging. In the first process, the same signal as that in the second process must not be generated. For example, the sequential excitation of two vibrational modes in a single molecule can be used to realize super-resolution Raman microscopy such as sum-frequency generation microscopy [28] and stimulated Raman scattering (SRS) microscopy [29,30]. Then, our technique can also be used to improve the sensitivity of molecular recognition. We believe that CSM microscopy will be an important tool in the future for investigating biological phenomena.

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