Saturated excitation of fluorescent proteins for subdiffraction-limited imaging of living cells in three dimensions

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We report, for the first time, the saturated excitation (SAX) of fluorescent proteins for subdiffraction-limited imaging of living cells in three-dimensions. To achieve saturation, a bright yellow and green fluorescent protein (Venus and EGFP) that exhibits a strong nonlinear fluorescence response to the high excitation intensity at the laser focus is used. Harmonic demodulation of the fluorescence signal produced by a modulated excitation light extracts the nonlinear fluorescence signals. After constructing the image from the nonlinear components, we obtain fluorescence images of living cells with spatial resolution beyond the diffraction limit. We also applied linear deconvolution to SAX microscopy and found it effective in further enhancing the contrast of small intracellular structures in the SAX image, confirming the expansion of the optical transfer function in SAX microscopy.

1. Introduction

Far-field optical microscopy is widely used for investigations in the life sciences, because it allows non-invasive and three-dimensional observations of biological specimens. However, spatial resolution is limited by the wave nature of light, which makes it difficult to resolve details in cells smaller than about half the illumination wavelength. In the past 20 years, the diffraction barrier has been overcome by several advanced techniques in far-field fluorescence microscopy such as stimulated emission depletion microscopy [1,2], photoactivated localization microscopy [3,4], stochastic optical reconstruction microscopy [5–7], saturated structured illumination microscopy [8–11] and so on. Recently, super-resolution fluorescence imaging of thick specimens was also achieved [12,13]. The key element common to these super-resolution techniques for breaking the diffraction limit is the use of the photoswitching property of fluorophores, which is realized by using various optical phenomena such as stimulated emission, cis–trans isomerization, triplet pumping [14] and saturated excitation (SAX) [15–17]. Structured illumination microscopy (SIM), while not breaking the confocal microscopy diffraction limit, is also well known as a high-resolution imaging technique which doubles the spatial resolution by overcoming the diffraction limit in classical wide-field fluorescence microscopy [18,19].

Recently, we used SAX of fluorescence to improve the spatial resolution of confocal fluorescence microscopy [20]. SAX microscopy exploits the nonlinear relationship between excitation and fluorescence intensity induced by saturating the population of fluorescence molecules at the excited state. SAX microscopy enables super-resolution imaging with a single excitation beam and is realized by a simple modification of a typical confocal microscope. Therefore, SAX microscopy can be applied to observe a wide range of...
specimens that are readily observable by confocal microscopy. In our past SAX experiments, we found that a high signal-to-noise ratio (SNR) in fluorescence detection is needed to extract the weak nonlinear fluorescence signals that contribute to the resolution improvement. This fundamental requirement explains why fluorescence imaging with SAX microscopy thus far has been demonstrated only with a fixed cell sample using immunostained fluorescence or fluorescent nanodiamonds [20–22].

In this paper, we report the first experimental result showing SAX of a fluorescent protein that allowed us to obtain subdiffraction-limited images of living cells by SAX microscopy. This success can be attributed to the use of a bright yellow and green fluorescent protein, Venus and EGFP [23], that provided sufficient SNR to extract the nonlinear fluorescence response induced by SAX under living cell conditions. Moreover, we demonstrate the further enhancement in the contrast of intracellular structures by simply applying linear deconvolution to the obtained SAX image and discuss the contrast enhancement by considering the effects of deconvolution on the optical transfer function (OTF) of SAX microscopy.

### 2. Saturated excitation microscopy

The underlying principle behind the resolution improvement in SAX microscopy is the exploitation of the nonlinear fluorescence response that emerges under SAX of fluorescence. Saturation occurs due to the non-zero fluorescence lifetime and the limited number of fluorescence molecules in the focal volume of the laser light. Because SAX predominantly occurs at positions with high excitation intensities such as at the centre of the laser focus, nonlinear fluorescence signals originate only from a region smaller than the diffraction-limited focal volume. Therefore, extraction of the nonlinear components gives a fluorescence image with a spatial resolution higher than the classical limit.

Extraction of the nonlinear fluorescence signals within the laser focus is, however, not straightforward, because, even at SAX conditions, linear fluorescence emission is still dominant. To extract the nonlinear fluorescence signals and separate it from the linear fluorescence signals, we use harmonic demodulation. The principle of harmonic demodulation is illustrated in figure 1. The excitation light is modulated temporally using a sinusoidal waveform, which produces an undistorted sinusoidal fluorescence waveform under no saturation conditions. When saturation occurs, the waveform of the fluorescence modulation becomes distorted, producing harmonic frequencies in the modulation (figure 1a,b). By applying a frequency filter, the nonlinear fluorescence signals (harmonic frequencies in the modulation) localized at the centre of the laser focus (figure 1d) can be extracted and used to construct a fluorescence image with subdiffraction-limited spatial resolution (figure 1c,d). Furthermore, the harmonic demodulation technique provides a simple way to further increase the spatial resolution of SAX microscopy.
microscopy by detecting higher harmonic frequencies, which originate from smaller regions in the laser focus.

Figure 1e shows the optical set-up for a SAX microscope. We used a continuous-wave (CW) laser with a wavelength of 488 nm (Coherent, Sapphire SF 488) and 532 nm (Cobolt, Samba) to excite the fluorophores. The laser intensity was modulated temporally by using two acousto-optic modulators (AOMs; Crystal Technology, 3080-125) arranged as shown. Briefly, the laser beam was split by a beam splitter. The separated beams passed through the AOMs, which induce a Doppler shift in the optical frequency of the laser beams after diffraction. The AOMs were driven at different frequencies (40.01 and 40.00 MHz) so that after superimposing the two diffracted laser beams, high SNR temporal sinusoidal modulation of the laser excitation intensity is achieved. Fluorescence signals from the samples were detected using a PMT (Hamamatsu, H7422-40), and harmonic demodulation of the signals was performed using a lock-in amplifier (NF Electronic Instrument, LI5640, or Zurich Instruments, HF2LI). To construct the fluorescence image, the laser focus was scanned along x-, y- and z-axis by using a two-axis galvano scanner (Cambridge technology, VM500) and a piezo stage (Physik Instrument, P-541.ZCD), respectively.

In addition to the fundamental resolution enhancement, SAX microscopy also improves the optical sectioning capability in practical conditions. In conventional confocal microscopy, the strong fluorescence signals from out-of-focus planes are reduced by detecting the signal through a finite-size pinhole. However, the pinhole cannot completely eliminate the out-of-focus signals, which can therefore still contribute to image formation resulting in decreased image contrast. In SAX, because the nonlinear fluorescence response originates locally in the laser focus, the contribution of fluorescence signals from out-of-focus planes to image formation is effectively suppressed. These results lead to the improvement of depth-discrimination property in a manner similar to two-photon excitation microscopy.

3. Nonlinear fluorescence response of fluorescent proteins

The successful application of SAX microscopy for high-resolution imaging of living cells depends essentially on a fluorophore that produces a high SNR fluorescence signals under SAX conditions. One possible candidate that we examined is a yellow fluorescent protein called Venus [23]. To determine the effectiveness of Venus for SAX microscopy, we investigated the fluorescence response of Venus under different excitation intensities. We measured the relationship between the excitation intensity and the demodulated fluorescence signal from Venus in HEPES buffer solution with a concentration of approximately 780 μM. The 532 nm excitation laser was focused into the solution with an NA-1.2 water-immersion objective lens. The excitation intensity was modulated at 10 kHz, and the fluorescence signals were demodulated at the fundamental frequency (10 kHz) and corresponding harmonic frequencies (20 and 30 kHz).

Figure 1f shows the plots of demodulated fluorescence signal intensity measured at various excitation intensities and detected at three demodulation frequencies (10, 20 and 30 kHz). The data confirm that Venus exhibits a second- and third-order nonlinear response measured at the second- and third-harmonic frequencies, and the behaviour of its fluorescence response is similar to those dyes reported in [21]. These measurements also suggest that the excitation intensity used for SAX imaging should be lower than approximately 30 kW cm⁻², because excitation intensities higher than this value could cause the harmonic signals to saturate, leading to degradation of the spatial resolution. In summary, the above results strongly indicate that Venus is a good fluorophore candidate for SAX microscopy of living cells.

4. Three-dimensional subdiffraction-limited imaging of a living cell

To demonstrate the spatial resolution improvement in the lateral axis (x–y plane), we observed living HeLa cells expressing Venus in mitochondria by using the SAX microscope with 532 nm CW laser, and the results are shown in figure 2a,b. In this observation, we used a silicone oil immersion objective lens of NA 1.3. The excitation intensity was 24 kW cm⁻². The excitation intensity was modulated at 10 kHz, and the fluorescence signals were demodulated at 20 kHz. Figure 2c shows the line profile of the structure indicated by arrows in figure 2b. The profile in figure 2c shows that SAX microscopy resolved the structure with a gap of around 130 nm, which is smaller than the diffraction-limited spatial resolution calculated from the NA and the wavelength (approx. 179 nm). The lateral resolution of SAX microscopy is theoretically calculated to be 136 nm. From this result, we consider that the achieved spatial resolution is close to the theoretical limit.

To compare the spatial resolution of SAX and conventional confocal microscopy, we observed the same sample with a confocal microscope, which was performed by the same set-up with reduced excitation intensity and demodulation at the fundamental frequency (10 kHz). The observed results are shown in figure 2d,e. The excitation intensity was 4 kW cm⁻² for this observation. The comparison of the obtained images clearly shows the improvement of the spatial resolution by SAX. The resolution improvement highlights the motion of the mitochondria. The difference of the spatial resolution is more evident in the enlarged images (figure 2f), where individual mitochondria are clearly separated.

We examined the improvement of axial resolution in SAX microscopy. Figure 2g and h are fluorescence images of a HeLa cell expressing Venus in mitochondria obtained with SAX. The experimental conditions were the same as those for figure 2a,d except for the pixel size and the pixel number. Comparison with the confocal images in figure 2i,k clearly shows the improvement of the axial resolution by SAX. The line profile shown in figure 2i confirms that SAX microscopy resolved the structure separated by approximately 360 nm gap along the axial direction, which cannot be recognized in the confocal image. The resolving power achieved in this experiment is close to the theoretical limit in SAX microscopy of 305 nm.

To test the possibility of the use of other fluorescent proteins in SAX microscopy, we also observed a HeLa cell expressing EGFP in Golgi apparatus. The x–y and x–z images are shown in figure 2m–p, respectively. The SAX and conventional confocal images were recorded simultaneously. For this observation, we used a 488-nm CW laser with an excitation intensity of 43 kW cm⁻². The results clearly show that
Venus and EGFP both provide sufficient SNR to perform high-resolution imaging in SAX microscopy.

We estimated the SNR in the raw image data obtained by SAX microscopy to show the signal strength of the nonlinear fluorescence response. It is not straightforward to calculate the SNR of SAX microscopy, and we estimated the SNR by the division of the total signal by the total noise (as defined by the area under the curve) in the optical transfer function.
5. Effect of deconvolution to saturated excitation imaging

The limitation of the spatial resolution in optical microscopy is described by the bandwidth of the OTF. SAX microscopy possesses a wider OTF than conventional confocal microscopy owing to the use of high-order nonlinear fluorescence response. However, the signal intensity at the low spatial frequencies is too strong to observe the high spatial frequency components in an image with high contrast. Linear deconvolution is an established technique to reduce the strength of the low spatial frequency components and enhance the contribution of the high spatial frequency components in an image.

We applied linear deconvolution to the SAX and confocal images (x–y plane) shown in figure 2a,d. The effective PSF for the deconvolution is calculated by using an electronic state model and the rate equations for the population probability of each level [24]. Figure 4b,c shows the deconvolved SAX images and the intensity line profiles of the structures before and after linear deconvolution. The valleys between the peaks are clearly deeper after deconvolution. This result clearly shows that deconvolution worked effectively to strengthen the high spatial frequency components in the image. We then compared the deconvolved SAX and confocal images. The deconvolved confocal images and the intensity profile are shown in figure 4c,f, respectively. Linear deconvolution also has been applied in confocal microscopy, and we confirmed that the spatial resolution in SAX microscopy remains higher than confocal imaging, even after linear deconvolution. This is attributed to the fact that SAX microscopy has an expanded OTF, rather than simply boosting high spatial frequencies.

We also examined the effectiveness of linear deconvolution in the x–z images (figure 2g,j). The deconvolved SAX and confocal images are shown in figure 4h,k, respectively. The comparison of the intensity profiles in figure 4i,l shows effective enhancement of the high spatial frequency components in the SAX image as well as the x–y images. The linear deconvolution was carried out using ImageJ (NIH, USA).

6. Conclusion and discussion

In this study, we showed the SAX of a fluorescent protein. The SNR of the protein images was sufficiently high to allow us to obtain for the first-time subdiffraction-limited images of a living cell by SAX microscopy. Using linear deconvolution techniques, the contrast of the SAX image was further enhanced. This enhancement was confirmed to be due to the increased contribution of high spatial frequency components.
components in the OTF of SAX microscopy. Hence, linear deconvolution enables us to fully use the performance of SAX microscopy for high-resolution imaging.

One key to this success was the choice of appropriate bright fluorescent proteins, Venus and EGFP. Fortunately, a number of other fluorescent proteins such as mTFP1 and mOrange2 are now available with similar properties. With brightness higher than or comparable with those of Venus and EGFP [25], these emerging fluorescent proteins provide high SNR and can be used to expand SAX microscopy targets.

One potential issue with the requirement of high excitation laser intensity for SAX microscopy is the higher rate of photobleaching compared with conventional confocal microscopy. The photobleaching effects can induce undesirable phenomena for living cells such as generation of reactive oxygen species, which are used in chromophore-assisted laser inactivation of proteins [26].

The effects of photobleaching on the sample can be reduced by using pulsed excitation, which is supported by several studies. For example, temporal separation of pulsed laser leads effectively to triplet relaxation [27]. Increasing the repetition rate of pulsed laser by pulse-splitting techniques also works to prevent photobleaching [28]. In the viewpoint of SAX microscopy, the use of pulsed laser excitation could allow us to induce sufficient saturated conditions while keeping the averaged laser power low because of the highly accumulated photons in a pulsed laser.

The analysis of the effect of deconvolution on the OTF of SAX microscopy revealed the effectiveness of combining deconvolution and OTF expansion for achieving higher spatial resolutions. This general approach could have many interesting applications. One idea is that it can be applied to increase the spatial resolution of conventional confocal microscope under SAX conditions by simply doing a linear deconvolution of the image. This is expected to work, because the OTF of conventional confocal microscopy under SAX condition is expanded and therefore has higher spatial frequency components beyond the diffraction limit.

Figure 4. Magnified views of fluorescence images shown in figure 2, and images after linear deconvolution. (a) SAX and (d) confocal images in x–y plane. (b) SAX and (e) confocal images after linear deconvolution. (c,f) The intensity profiles of the structures indicated by arrows in panels (a,b) and (d,e). (g,j) SAX and confocal images in x–z plane. (h,k) Images after linear deconvolution. (i,l) the intensity profiles of the structures indicated by arrows in (g,h) and (j,k).
The advantage of SAX microscopy compared with the other super-resolution techniques is the three-dimensional high-resolution imaging capability of a thick sample, such as cell clusters and tissues. In SAX microscopy, fluorescence signals from out-of-focus planes are effectively eliminated in the same manner as two-photon excitation fluorescence microscopy, resulting in the enhancement of the spatial resolution in a thick sample.

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