# High-Resolution Confocal Microscopy by Saturated Excitation of Fluorescence

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We demonstrate the use of saturated excitation in confocal fluorescence microscopy to improve the spatial resolution. In the proposed technique, we modulate the excitation intensity temporally and detect the harmonic modulation of the fluorescence signal which is caused by the saturated excitation in the center of the laser focus. Theoretical and experimental investigations show that the demodulated fluorescence signal is nonlinearly proportional to the excitation intensity and contributes to improve the spatial resolution in three dimensions beyond the diffraction limit of light.

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The advantage of using optical microscopes to image biological samples is that the specimens can be observed in living conditions. Although the spatial resolution cannot compete with that of electron microscope techniques, optical microscopes allow us to observe a living sample *in vivo*, and without causing significant damage to the sample. In addition, *in vivo* imaging by optical microscopy allows a variety of biological phenomena to be observed. Fluorescence microscopy, in combination with various fluorescence probes, can be used to visualize fundamental cellular processes such as expression of genes, ionic concentration change, and membrane potential change [1,2].

To further explore biological phenomena while retaining the advantages of the optical microscope, various techniques have been attempted to improve the spatial resolution of the microscope. Particularly, in recent developments of high-resolution fluorescence microscopes, saturation phenomena in optical effects have been used to circumvent the diffraction limit of light. Since the saturation imposes strong nonlinearity in the relation between excitation rate and fluorescence emission, it can be used to achieve spatial resolution beyond the diffraction limit. Stimulated emission depression (STED) microscopy uses the saturation effect in depression of fluorescence emission to reduce the size of the region of fluorescence emission [3], and has been applied to fluorescence nanoimaging of biological samples [4,5]. The spatial resolution of about 16 nm has been demonstrated [6] by STED microscopy. Photoswitchable fluorescent protein and the saturation effects in photoswitching have been used to reduce the size of the fluorescence emission region down to 100 nm in the lateral direction [7]. Use of saturated excitation in structured-illumination microscopy has also been proposed [8], and a spatial resolution of about 50 nm has been demonstrated [9].

In this Letter, we demonstrate the use of saturated excitation to improve the spatial resolution in laser scanning confocal microscopy. In conventional fluorescence microscopy, the fluorescence intensity given from a sample is proportional to that of the excitation intensity. However, as the excitation intensity is increased, population of fluorescence molecules at the excitation level shows saturation because the molecules have a nonzero excitation lifetime, and the number of molecules in the focal volume is limited. In this condition, the fluorescence intensity is no longer linearly proportional to the excitation intensity, and the point-spread function (PSF) of fluorescence is deformed from that given in unsaturated conditions [10]. Since the PSF defines the region of fluorescence emission, deformation of the PSF usually flattens and widens the profile of fluorescence emission, decreasing the resolution. Excitation saturation has therefore been avoided in laser scanning fluorescence microscopy [11]. However, the deformed PSF contains high spatial frequency components, which are created when the fluorescence saturation introduces strong nonlinearity in the excitation-emission relationship. Since these high spatial frequency components occur first at the peak of the laser excitation, they carry structural information on a scale that is smaller than the PSF and thus, may be used to improve the three-dimensional spatial resolution of the microscope.

To extract the nonlinear components in fluorescence emission that contribute to improvement of the spatial resolution, we temporally modulate the excitation intensity at a frequency ($\omega$) and demodulate the fluorescence intensity at the corresponding harmonic frequencies ($2\omega$, $3\omega$, ...). When the excitation intensity is high enough to generate saturation in fluorescence excitation, harmonic frequencies appear in the modulated fluorescence signal because the relationship between fluorescence and excitation intensity becomes nonlinear. This effect can be seen prominently at the center of the PSF of excitation, and can be used to detect fluorescence intensity at the position close to the center of PSF.

We calculated the fluorescence signal that can be obtained by the harmonic demodulation. We used a photophysical model with five electronic levels and rate equations of population probabilities in each levels pre-

 

sented in Ref. [12]. We solved the rate equations for steady-state population probabilities analytically by using the state transition matrix under the assumption in which the modulation period is sufficiently longer than the fluorescence lifetime. We also assumed that there is no photobleaching during the cycles of excitation and emission of fluorescence. We calculated fluorescence intensity at various excitation intensities in the modulation. Fluorescence signals demodulated at the fundamental frequency ($\omega$) and the harmonic frequencies ($2\omega$, $3\omega$, ...) were obtained by performing Fourier transform of the calculated fluorescence signal as shown in Fig. 1. The calculation has been performed for Rhodamine 6G molecules excited by a cw laser of which wavelength was 532 nm. Photophysical parameters of Rhodamine 6G shown in [12] were used for the calculation.

We found that the fluorescence signal obtained by the harmonic demodulation exhibits a nonlinear response to the excitation intensity, and the order of the nonlinearity increases with the frequency of demodulation. The fluorescence signal given by demodulation at the frequency of $n\omega$ ($n = 2, 3, \ldots$) is proportional to the $n$th power of the excitation intensity at low excitation intensity, and shows saturation at high excitation intensity. From these results, we conclude that the spatial resolution can be improved in three dimensions by choosing the excitation intensity and the frequency for harmonic demodulation so that the chosen harmonics does not show saturation. For example, by choosing the 3rd-order harmonic signal that is proportional to the cube of the excitation intensity, the resulting spatial resolution can be twice as high as that of a typical confocal fluorescence microscope. Since the higher-order harmonic signal extracts the higher-order nonlinear response between fluorescence excitation and emission, the improvement of spatial resolution in the proposed technique is theoretically unlimited.

We also calculated effective PSFs of confocal fluorescence microscopy with saturated excitation. A PSF of confocal fluorescence microscopy, $h_{\mathrm{conf}}$, is given as [13],

$$h_{\mathrm{conf}}(x, y, z) = h_{\mathrm{ill}}(x, y, z)h_{\mathrm{det}}(x, y, z), \tag{1}$$

where $h_{\mathrm{ill}}$ and $h_{\mathrm{det}}$ are an illumination PSF and a detection PSF, respectively. To obtain an effective PSF with saturated excitation, $h_{\mathrm{conf\text{-}sat}}$, the illumination PSF has to be replaced with a PSF of demodulated fluorescence, $h_{\mathrm{dem}}$, since the distribution of detectable fluorescence intensity is not a linear projection of $h_{\mathrm{ill}}$ and varies with the frequency of demodulation.

$$h_{\mathrm{conf\text{-}sat}}(x, y, z) = h_{\mathrm{dem}}(x, y, z)h_{\mathrm{det}}(x, y, z). \tag{2}$$

$h_{\mathrm{dem}}$ can be obtained by calculating demodulated fluorescence intensity as mentioned above with excitation intensity given by $h_{\mathrm{ill}}$ [14]. We obtained effective PSFs with saturated excitation with various demodulation frequencies as shown in Fig. 2. The calculation has been performed for a sample with Rhodamine 6G excited by using a cw laser with the wavelength of 532 nm and a water-immersion objective lens of which the numerical aperture (NA) was 1.2. The wavelength of the emission was assumed to be the same as that of the excitation. The calculated results show that the spatial resolution can be improved in both lateral and axial directions, and a higher spatial resolution can be obtained with a higher demodulation frequency.

We experimentally confirmed the improvement of the spatial resolution. We used a standard optical setup for confocal fluorescence microscopy equipped with a laser-intensity modulator and a lock-in detection system. We

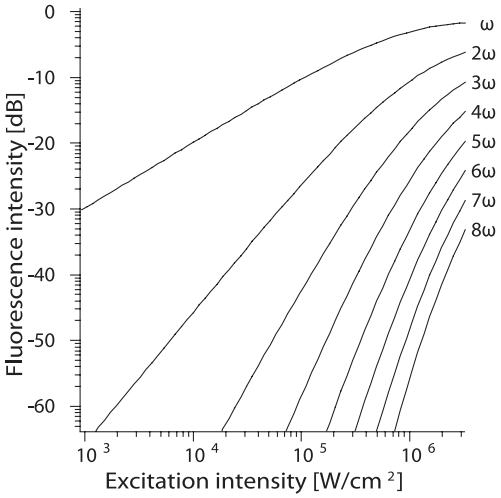


FIG. 1. The calculated relationship between the excitation intensity and the demodulated fluorescence signals. The higher demodulation frequency gives a higher order of nonlinear response, resulting in higher spatial resolution of the microscope. The lateral axis shows the average of the modulated intensity.

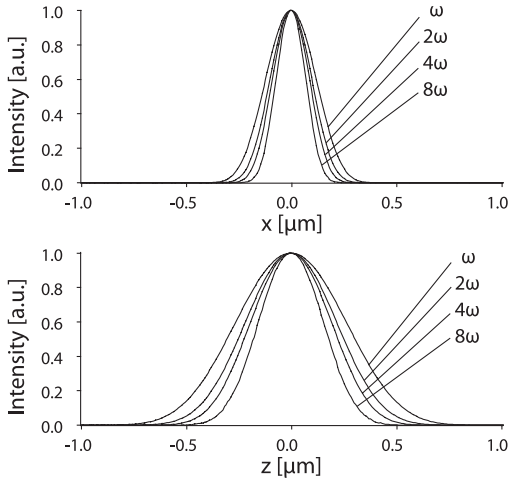


FIG. 2. Profiles of the effective PSFs on the lateral ($x$) and axial ($z$) axis calculated from the demodulated fluorescence intensities. Excitation intensities for demodulation frequencies $\omega$, $2\omega$, $4\omega$, and $8\omega$ are 1.5, 15, 400, and 700 kW/cm$^2$, respectively. With these excitation intensities, no evident saturation is seen for each demodulated signal in the calculated excitation-emission relationships.

used a cw solid-state laser (uniphase, wavelength: 532 nm) for the source of fluorescence excitation. Modulation of the laser intensity was made by using two acousto-optic modulators (AOMs; IntraAction, AOM-40) driven by two different frequencies, 40.000 MHz and 40.010 MHz. The laser beam was divided into two by a beam splitter and incident to the AOMs separately. By being diffracted at the AOMs, two laser beam obtained a doppler shift in optical frequency by the AOM-driving frequency. The diffracted beams were superimposed by using a beam splitter, and a sinusoidal modulation in laser intensity was made by interference of light. Fluorescence from a sample was detected by a photomultiplier tube (Hamamatsu, H7422-40) through a dichroic mirror, a low pass filter, and a pinhole with the diameter of 25 $\mu$m which corresponds to the diameter of the Airy disc at the pinhole. The harmonic signal was obtained through a lock-in amplifier (NF Electronic Instrument, LI5640). The fluorescence signal was also analyzed by a digital oscilloscope (Agilent, Infiniium).

The demodulated fluorescence intensity was measured with various excitation intensities to confirm that the nonlinear response of the signal was induced by the saturated excitation. Figure 3 shows the frequency of modulated fluorescence signal from a Rhodamine 6G solution with the concentration of 10 $\mu$M excited by a water-immersion objective lens of which the NA was 1.2. We confirmed that the 2nd and 3rd harmonic distortion appeared in the fluorescence signal at high excitation intensity. Figure 4 shows the fluorescence intensity measured with the lock-in amplifier using a demodulation frequency of $\omega$ (10 kHz), $2\omega$ (20 kHz) and $3\omega$ (30 kHz). In Fig. 4, the fluorescence signal given at the 2nd-harmonic frequency ($2\omega$) is proportional to the square of the excitation intensity, whereas the signal given at the fundamental frequency ($\omega$) is almost proportional to the excitation intensity. This result shows that the nonlinear response given by the harmonic demodulation can be used to improve the spatial resolution of the microscope. This also confirms that the signal with the frequency of $2\omega$ is given by the excitation saturation and not by a nonlinear response originating from harmonic distortion of the laser modulation because a harmonic signal arising from the distortion of the laser modulation linearly proportional to the excitation power. The linearity of the detection system was also confirmed by detecting the modulated laser intensity by the PMT. We also confirmed fluorescence signal with the 3rd-harmonic frequency. Although the 3rd-harmonic signal shows nonlinear response to the excitation intensity, the signal to noise ratio (S/N) was not sufficient to use the signal for improving the spatial resolution. The main source of the noise is the shot-noise in the PMT, which is distributed in any frequency of the lock-in detection. In this experiment, the shot-noise can be seen as the signal increasing with the slope of 0.5 in the 3rd-harmonic signal. We also examined solutions of Rhodamine B (532 nm excitation), Fluorescein (488 nm excitation) and Alexa 488 (488 nm excitation). These experimental results also showed fluorescence responses similar to Fig. 4.

We observed fluorescence beads with a diameter of 200 nm (Invitrogen, Fluospheres (540/560)) fixed on a substrate in water. Figures 5(a) and 5(b) show the fluorescence images of the sample. We modulated the excitation intensity at 10 kHz, and demodulated the detected fluorescence signal at the fundamental (10 kHz) and the 2nd harmonic (20 kHz) frequencies for Figs. 5(a) and 5(b), respectively. The average power of excitation for Figs. 5(a) and 5(b) at the focus were 3 and 14 $\mu$W corre-

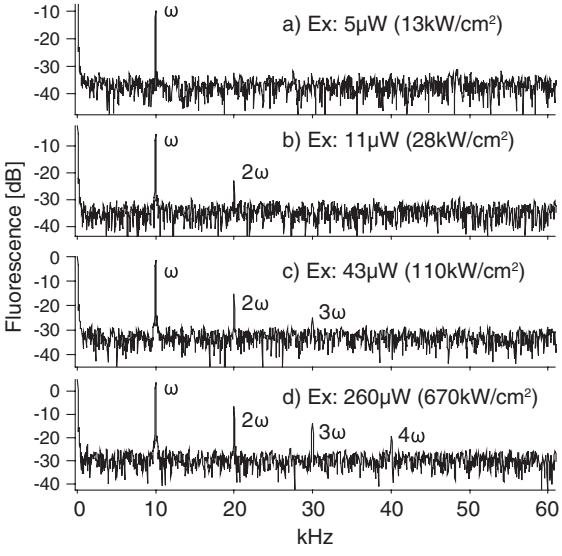


FIG. 3. Frequency components in fluorescence signal excited with various excitation power. A Rhodamine 6G solution with the concentration of 10 $\mu$M was used as the sample. The frequency of the excitation modulation was 10 kHz.

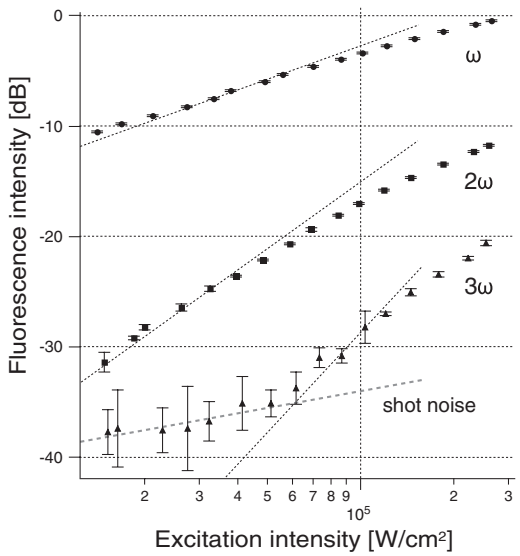


FIG. 4. Fluorescence intensity obtained by demodulating fluorescence signal from a Rhodamine 6G solution at 10, 20, and 30 kHz. The frequency of the excitation modulation was 10 kHz.

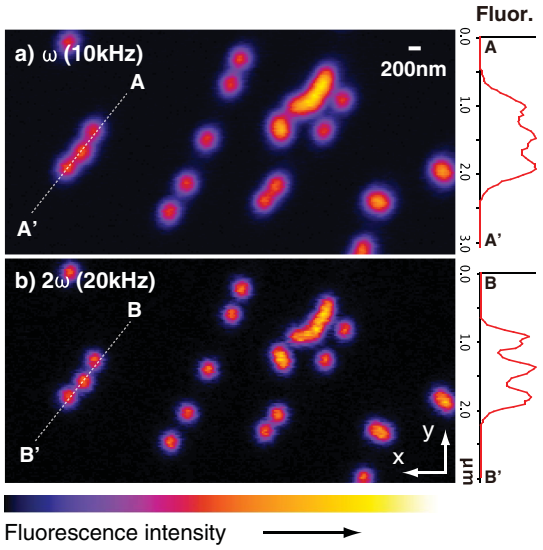


FIG. 5 (color). Fluorescence images of fluorescence beads were obtained by demodulation at (a) the fundamental and the (b) 2nd-harmonic frequency. The diameter of a bead is 200 nm.

sponding to excitation intensities of 7.8 and 36.4 $kW/cm^2$, respectively. The pixel dwell time for both images was 0.2 ms. We chose a relatively short pixel dwell time to prevent photobleaching by long exposure. Three images were taken at the same position of the sample and were averaged. The gaps of the beads can be recognized more clearly by the 2nd-harmonic signal. From the comparison of these images, the improvement of the spatial resolution with the 2nd-harmonic demodulation is confirmed.

In this Letter, we described the use of saturated excitation in laser scanning confocal microscopy to improve the spatial resolution. The results of theoretical calculations show that higher spatial resolution can be obtained by demodulation at a higher-order harmonic frequency. The spatial resolution of this technique is not limited theoretically, but rather by the $S/N$, the dynamic-range of the detection system and the nonlinearity inherent in the light illumination and detection system. This means that the spatial resolution can be improved further by development of detection system with high $S/N$ and wide dynamic range in the future.

One of the advantages of our technique compared to other types of high-resolution optical microscopy is the simple optical system. Adding a laser-intensity modulator and a lock-in amplifier into a typical confocal fluorescence microscope are the main modifications required to realize the proposed microscope. The use of confocal detection also has advantages in observation of thick samples, where $S/N$ and signal-to-background ratio are improved by factors of 10 and 100, respectively [15].

Since the efficient detection of the harmonic components in fluorescence requires high excitation intensity, photobleaching is a main problem for applying the technique for biological observation. This disadvantage limits the application of this technique to observation of samples with bright and stable fluorescence molecules. Using a pulsed laser is effective to reduce the photobleaching effect because accumulated photons in a laser pulse easily saturate the excitation without increasing the total number of incident photons. This also helps to increase number of fluorescence photons emitted before photobleaching and to improve the $S/N$ of fluorescence detection [16]. In Fig. 4, the shot-noise apparently limits the detectable nonlinear signals. This problem may be reduced by using a shot-noise free detector, such as a high-gain avalanche photodiode if the fluorescence signal is large enough.

The technique described here can also be applied to multiphoton microscopes [17] and also in near-field scanning optical microscopes for the further improvement of spatial resolution [18,19]. Multiphoton excitation helps to reduce photobleaching at out-of-focus planes. It is also possible to detect nonlinear optical effects hidden in strong linear effects. Nonlinear optical effects appear at higher harmonic frequencies in a detected signal, and can be separated from linear effects. This has been applied to measure two-photon absorption cross section of Rhodamine 6G directly from the laser power transmitted through the sample [20].

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