

Imaging properties of saturated-excitation fluorescence microscopy

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KEY WORDS: Fluorescence microscopy, confocal, saturated excitation.

The spatial resolution of confocal fluorescence microscope is limited to about half a wavelength, because light has a wave-like nature and cannot be focused into a dimensionless spot. The broadened focused light spot excites the fluorescence sample and causes a broadened distribution of fluorescence emission. The distribution of fluorescence emission is the same as the excitation distribution when the emission intensity of fluorescence is proportional to the excitation intensity. If, however, the emission intensity of fluorescence is proportional to the square or cube of the light intensity, the distribution of fluorescence emission may become reduced in size. The small spot of fluorescence emission can achieve high spatial resolution confocal fluorescence microscopy.

We developed a high-resolution laser-scanning confocal fluorescence microscopy based on nonlinear fluorescence emission under saturated excitation[1]. This technique uses modulated excitation to drive the fluorescent response into saturation, whereby nonlinear emission components can be extracted. At saturation, fluorescent molecule emission intensity exhibits a high-order nonlinear dependence on the excitation intensity, when the molecule is excited by high intensity light. This nonlinear fluorescence response was analyzed by rate equations formulated from a five-level system Jablonski Diagram. The high-order nonlinearity can be obtained by modulation of the excitation intensity at frequency ω by using the interference of two acousto-optic modulators and corresponding demodulation of fluorescence intensity harmonics at integer multiples of the fundamental frequency ω . Fig. 1 shows the calculation result of the response of rhodamine-6G which is irradiated by 532nm CW light. The imaging properties of a confocal microscope in a partially saturated condition are given by $h_{conf-sat}(\mathbf{r}) = h_{fl}(\mathbf{r}) \bullet h_{det}(\mathbf{r})$ where $h_{fl}(\mathbf{r})$ is the fluorescence intensity distribution near the focus and $h_{det}(\mathbf{r})$ is the point spread function (PSF) determined by the detection system. Fig. 2 shows several PSFs obtained by calculating the fluorescence distribution near the focus. From fig. 2, higher harmonic components produce a smaller PSF and provide higher spatial resolution. The optical transfer function can be calculated from the PSF. As a result, the $n\omega^{\text{th}}$ harmonic frequency of the fluorescence signal gives n times higher spatial resolution.

Acknowledgement: This study was supported by Industrial Technology Research Grant Program in 2006 from New Energy and Industrial Technology Development Organization (NEDO) in Japan.

[1] K. Fujita, et. al., *Phys. Rev. Lett.* **99**, 228105 (2007).

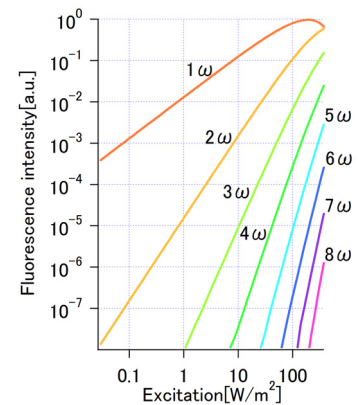


Fig. 1; Fluorescence intensity of harmonic components

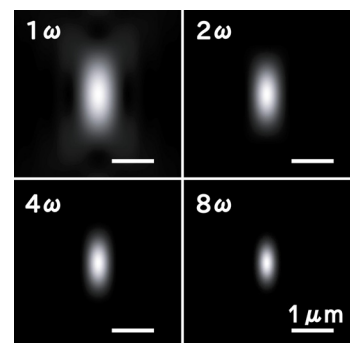


Fig. 2; PSF of harmonic components