Spatial resolution improvement in confocal fluorescence microscopy by using saturated excitation

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Confocal fluorescence microscopes are indispensable tools for scientific investigations in biology and medicine, since it provides a noninvasive, 3-dimensional imaging of biological samples in their living conditions. In fluorescence microscopy, the spatial resolution for imaging is subject to the diffraction-limited spot size of focus. This limitation is given on the assumption that a distribution of fluorescence intensity in the focal spot is a linear projection of the excitation intensity.

We developed a high-resolution laser-scanning confocal fluorescence microscope based on nonlinear fluorescence emission under saturated excitation [1]. When fluorescence molecules are excited with high intensity light, the excited state population shows saturation because of the existence of a lifetime for fluorescence. Under this condition, the relationship between excitation intensity and fluorescence intensity becomes nonlinear. In the region close to the center of the focal spot, saturation of the excited state can occur easily with high excitation intensity. By detecting the nonlinear components of fluorescence emission, which appear at the center of the focus, the spatial resolution can be improved. To extract these nonlinear components of fluorescence emission, we modulated the excitation intensity temporally at a frequency (ω) and demodulated the fluorescence intensity at the harmonic frequencies (2ω, 3ω,…). We observed fluorescence microspheres with a diameter of 200 nm (Invitrogen, Fluospheres (540/560)) fixed on a substrate in water. Fig.1 a) and b) show images of the fluorescence microshperes and the intensity profiles spanning the dotted lines shown in these images. In this observation, we modulated the excitation intensity at 10kHz, and demodulated the fluorescence intensity at the fundamental frequency and the 2nd harmonic frequency (20kHz). We used a CW solid-state laser (uniphase, wavelength: 532nm) as the excitation laser source and a water-immersion objective lens (NA 1.2, ×60) for excitation and detection of fluorescence. In our results, the gap between two fluorescent microspheres was more clearly imaged with the fluorescence signal given by the 2nd harmonic frequency than with the fundamental. From these results, we confirmed the improvement of spatial resolution by the method we developed.

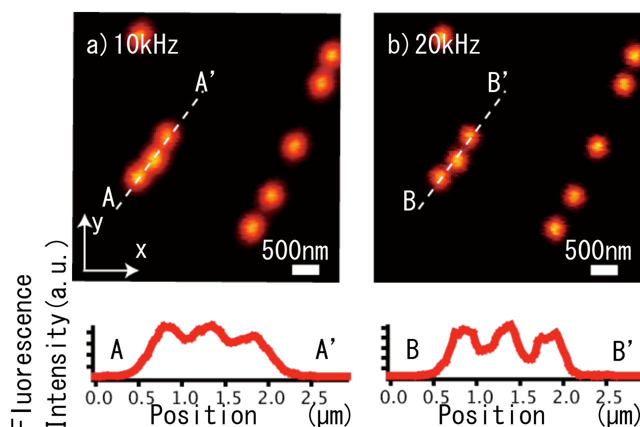

Fig.1 Images of fluorescence microsphere obtained by demodulation at a) the frequency (10kHz) and b) the 2nd harmonic frequency(20kHz). The plot shown in the bottom of the images shows the intensity profile of the dotted lines in the images.

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Reference: [1] Fujita, Yamanaka et al., Phys. Rev. Lett, 99, 228105 (2007)