Saturated excitation microscopy with extracting nonlinear fluorescence signals by signal subtraction

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The recent development in super resolution microscopy achieved the spatial resolution beyond the diffraction limit of light and has provided powerful tools for biological studies [1]. The key techniques for breaking the diffraction barrier were from the understanding and utilizing the interaction between photons and fluorescence molecules.

Previously we have proposed saturated excitation (SAX) microscopy as a technique for achieving super resolution imaging in confocal microscopy, which utilizes the excitation saturation of the fluorescence molecules [2]. Higher intensity excitation induces saturation of fluorescence signal from a sample, inducing nonlinear relationships between the fluorescence excitation and emission. Since the excitation saturation occurs predominantly within the focal spot, extracting the nonlinear fluorescence signal provides fluorescence images with a spatial resolution higher than the diffraction limit. The extraction of nonlinear fluorescence signals has been achieved by modulating excitation intensity and demodulated fluorescence signals at harmonic frequencies. We demonstrated SAX microscopy in observation of various fluorescence molecules such as fluorescence proteins and dyes [3, 4]. We also analyzed the relationship between the efficiency of excitation saturation and the modulating frequency of excitation for three dimensional imaging with reduced photobleaching [5,6]. SAX microscopy has also been applied to observation of non fluorescence samples [7, 8].

We report another technique to extract nonlinear fluorescence signals from saturated fluorescence, which improves signal-to-noise ratio (SNR) and spatial resolution in SAX microscopy. In this method, nonlinear signals can be extracted by subtracting of non-saturated and saturated fluorescence signals, without modulating excitation. This subtraction-based SAX (S-SAX) microscopy can theoretically detect 8 times and 32 times more signals than modulating SAX (M-SAX) microscopy, in detection of 2nd order, and 3rd order of nonlinear fluorescence responses, respectively. We also optimized excitation conditions for efficiently induction of fluorescence saturation, that allows the improvement of SNR due to reduced light irradiation and photobleaching. With the improved SNR, extraction of higher order of nonlinear fluorescence signals is possible. We demonstrated the improvements of spatial resolution in observing fluorescent polystyrene beads, actin filamentous structures in stained HeLa cells, and synapses in sliced and stained mouse brain tissue.

Figure 1 shows the fluorescence images of synapse reconstructed with confocal (linear), 2nd order, and 3rd order nonlinear fluorescence signals. For extracting 2nd and 3rd order S-SAX images, two and three confocal images with different excitation intensities were required. Each image was obtained with an averaged excitation power of 0.35, 1.32, and 3.73 kW/cm² (for the 3rd order image) through an oil immersion objective (x60, NA1.42). Pixel size and irradiation dwell time for each image were 51 nm and 100 μs, respectively.

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References