SAX microscopy with fluorescent nanodiamond probes for high-resolution fluorescence imaging

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Abstract: We report the use of fluorescent nanodiamonds (FNDs) as a photostable fluorescent probe for high resolution saturated excitation (SAX) microscopy. We confirmed that FNDs show a nonlinear fluorescence response under saturated excitation conditions generated by intense excitation light. Using FNDs, we quantified the spatial resolution improvement inherent in SAX microscopy, and experimentally demonstrated the scalability of the spatial resolution of SAX microscopy. The photostability of the FNDs allowed us to perform nanoparticle imaging of a multicolor-stained macrophage cell with a spatial resolution beyond the diffraction limit.

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OCIS codes: (170.1790) Confocal microscopy; (170.2520) Fluorescence microscopy.

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1. Introduction

In optical microscopy, the spatial resolution is restricted by the diffraction limit of light. In the last two decades, however, new concepts in fluorescence microscopy were applied to overcome the limitation, including stimulated emission depletion (STED) microscopy [1,2], photoactivation localization microscopy (PALM) [3], fluorescence PALM (FPALM) [4] and stochastic optical reconstruction microscopy (STORM) [5]. The key element of the emerging high-resolution techniques is photoswitching of fluorophores. The use of optical phenomena such as stimulated emission, photoactivation, cis-trans isomerization, triplet pumping [6], and saturated excitation [7–11] are major ways to realize this photoswitching.

Recently we have reported the use of saturated excitation (SAX) for improving the spatial resolution of confocal microscopy [12,13]. SAX microscopy exploits the nonlinear fluorescence response which occurs when fluorescence molecule excited states become saturated. The saturation appears predominantly in the center of the laser focus and the nonlinear signals can be extracted by temporal modulation of the excitation intensity at a single frequency and harmonic demodulation of the emitted fluorescence signals. Thus, a SAX microscope can be realized by simple modification of a typical confocal setup; the introduction of an incident laser modulator and a lock-in amplifier detection path are sufficient. In SAX microscopy, higher harmonic demodulation gives higher-order nonlinear fluorescence signals that appear from a region smaller than the excitation point spread function (PSF), so that the resolution enhancement is theoretically unlimited [14]. Practically, a major limitation of the spatial resolution in SAX microscopy occurs due to photobleaching effects, since an intense excitation intensity is required to induce sufficient saturated excitation, which is also true in other high-resolution techniques requiring high excitation intensity [7–11].

In this paper, we report the use of fluorescent nanodiamonds (FND) as a photostable fluorescent probe for SAX microscopy. FNDs do not show photobleaching because the fluorescence results from point defects inside a solid matrix of the diamond [15]. FNDs allow us to observe biological specimens without photobleaching problems, and are also useful to obtain practical measurements of the imaging properties of SAX microscopy. FNDs can also be used for STED microscopy, where a resolution of up to 5.8 nm has been reported [16].
FND can be bound to proteins through antigen-antibody interactions for fluorescence imaging of protein distributions in a cell [17].

We used FNDs with negatively charged nitrogen-vacancy defect centers, (N-V), which is one of the point defect modes in diamonds (Fig. 1 a) [18]. The FND of this type absorbs light at ~532 nm, is excited from the ground triplet state \((^3A)\) to the excited triplet state \((^3E)\), and then emits fluorescence from 550 to 800 nm. The fluorescence lifetime is 11.6 ns and the quantum efficiency is ~0.7 [19]. In the excitation and fluorescence emission process, intersystem crossing to the single excitation state \((^1E)\) may also occur resulting in non-radiative decay to the ground state.

![Fig. 1. (a) Structure of the nitrogen-vacancy defect center in diamond and (b) the energy level scheme](image)

2. **Nonlinear fluorescence response of FNDs**

We calculated fluorescence signal intensities using the electronic state model shown in Fig. 1 b) and the rate equations for the population probability of each level. In this calculation, we treated \(^1E\) and \(^1A\) as a single level [20] because the lifetime of \(^1E\) (~30 ns) is almost 10 times shorter than the time-scale for non-radiative decay from \(^1A\) to \(^3A\) (~300 ns). The other parameters of absorption cross-section \((10^{-16} \text{ cm}^2)\), intersystem crossing rate \((30 \times 10^6 \text{ s}^{-1})\), and the non-radiative decay rate from \(^1A\) to \(^3A\) \((3.3 \times 10^6 \text{ s}^{-1})\) are from references 21 and 22.

The rate equations were solved by the state transition matrix to estimate the fluorescence signal with various excitation intensities in the modulation. Demodulated fluorescence signals were given by the Fourier transform of the calculated fluorescence signal [14].

Figure 2 shows the calculated demodulated fluorescence intensities with various excitation intensities under the excitation intensity modulation fundamental frequency \((f_m)\) of 10 kHz. The demodulation frequencies were the \(n\)th harmonics of the fundamental \((n f_m\) where \(n = 2, 3, 4, \ldots\)). In the results, we confirmed that FNDs show saturation in fluorescence excitation,
and demodulated fluorescence signals at $n$th harmonic frequencies show corresponding $n$th order nonlinear fluorescence responses ($n = 2, 3, 4, \ldots$), in a similar manner to fluorescent dyes we have previously demonstrated [13].

3. Improvement of the spatial resolution by SAX microscopy

Based on the results in Fig. 2, we also calculated the effective point spread functions (PSFs) obtained by confocal (Fig. 3 a)) and SAX microscopy (Figs. 3 c), d), and e)). For the calculations, we used the same parameters that were used in Fig. 2. The effective PSF for SAX microscopy is given by the product of a detection PSF and a PSF of demodulated fluorescence [12,14]. For the calculation, an objective lens with an NA of 1.4 was used for both sample illumination and fluorescence detection. The size of the pinhole for confocal detection was assumed to be infinitely small. As shown in Fig. 3, the size of the effective PSF becomes smaller when higher demodulation frequencies are used. From these calculation results, we confirmed theoretically that the FNDs can be used as a fluorescent probe for high-resolution imaging in SAX microscopy.

![Fig. 3. The calculated effective PSFs for fluorescence signals demodulated at the fundamental ($f_m$) and the harmonic frequencies ($2f_m$, $3f_m$, and $4f_m$). The excitation intensities for $f_m$, $2f_m$, $3f_m$, $4f_m$ are 3.5, 9, 17, 25 kW/cm$^2$, respectively.](image)

4. Measurement of nonlinear fluorescence emission from FNDs

We experimentally measured demodulated fluorescence signal intensities from a FND with various excitation intensities (Fig. 4). The excitation light source was a continuous-wave (CW) laser with a wavelength of 532 nm, and the laser was focused into a FND with an oil-immersion objective lens (N.A. = 1.45). We used a FND with a diameter of approximately 100 nm. The excitation intensity was modulated at a fundamental frequency of 10 kHz. The fluorescence intensity was demodulated at the fundamental and corresponding harmonic frequencies (20, 30, 40 kHz).

As indicated from the calculations shown above, the measurement shows the nonlinear relation between the excitation intensity and the demodulated fluorescence signals. It was also observed that the order of nonlinearity in the response increased with higher demodulation frequencies. The nonlinear fluorescence response confirms that the FND can be saturated by a high excitation intensity and is a suitable probe for improving the spatial resolution by SAX microscopy. We also observed a saturation effect in the demodulated signals, as predicted by calculations in Fig. 2, indicating that the spatial resolution may degrade if we apply an excessive excitation intensity. The excitation intensity required to observe harmonic signals with the FND is comparable with that for typical fluorescent molecules, such as Rhodamine
6G. Although the lifetime of the FND is longer than that of the dye, the lower absorption-
cross section of the FND requires a higher excitation intensity to compensate and produce a
detectable saturation effect. The excitation intensity needed in the experiments was higher
than that predicted in the theoretical investigation. This is presumably due to the difference
between the photophysical parameters of the sample FND and those used in the calculations.
The difficulty of estimating the local excitation intensity at the FND location in the small
focal volume may also contribute to the difference. Aside from the disagreement in the
excitation intensity, this experiment result confirms that FNDs can be used as practical
fluorescence probes for SAX microscopy.

![Graph showing the relationship between FND demodulated fluorescence and excitation intensity.](Image)

**Fig. 4.** Experimentally measured relationship between FND demodulated fluorescence and excitation intensity. The modulation frequency of the excitation laser was 10 kHz. Dotted lines show the gradients of slopes of 1, 2, 3, and 4, as viewed from the left side of the graph.

### 5. Measured point spread functions of SAX microscopy

We imaged a single FND fixed on a coverslip to observe the scalability of the spatial
resolution of SAX microscopy with different demodulation frequencies. The mean size of the
FND was 100 nm in diameter. In the experiment, the FND was excited with a CW laser of
532 nm wavelength and an oil-immersion objective lens (N.A. = 1.4). The excitation laser was
modulated at 10 kHz and fluorescence signals were demodulated at the fundamental (10 kHz),
second harmonic (20 kHz), third harmonic (30 kHz), and fourth harmonic (40 kHz)
frequencies. The excitation intensities for these frequencies were 3.5, 9.0, 17 and 25 kW/cm²,
respectively. The pixel dwell times were 10 ms for the fundamental and 50 ms for the second,
third, and fourth harmonic frequency, and the images were 35 × 35 pixels.

Figure 5 shows the fluorescence images obtained by conventional confocal (Fig. 5 a)) and
SAX microscopy (Figs. 5 b), c), and d)). The fluorescence intensity line profiles along the
dotted lines in the images are also shown in Fig. 5. Comparing Fig. 5 a) and b), we confirmed
that higher spatial resolution is achieved in three-dimensions by demodulation of fluorescence
signals at the second harmonic frequency. Further improvement of the three-dimensional spatial
resolution can be obtained by demodulating at the third and fourth harmonic frequencies (Fig. 5 c) and d)). In the fluorescence images, the image size of the FND becomes smaller with higher demodulation frequency, indicating the improvement of the spatial
resolution in three dimensions. Since the actual distribution of color centers in the FND is
unknown, the effective PSF of the optical system cannot be estimated from these results.
However, this experiment clearly demonstrates the scalability of the SAX microscopy spatial
resolution by changing the demodulation frequency.
6. Photostability of FNDs under SAX microscopy

As pointed out in previous reports [15,16], FNDs exhibit strong photostability even under intense light illumination. To test the photostability under saturated excitation conditions, we took multiple sequential fluorescence images of one single 100 nm FND with SAX microscopy. Fluorescence images were taken sequentially 8 times, using the third harmonic signal demodulation (Fig. 6). As shown in the results, we confirmed that photobleaching was not observed. In this experiment, the excitation intensity was 61 kW/cm$^2$, the pixel dwell time was 2 ms, and the pixel size was $35 \times 35$. 

Fig. 6. Images of a single FND observed by SAX microscopy with the third harmonic demodulation ($3f_m$, 30 kHz). Each image (1-8) was obtained sequentially from the FND with a diameter of 100 nm.
7. Dual-color imaging of FNDs and mitochondria in macrophages

Particle-based imaging techniques are widely utilized for investigations of cellular processes such as intracellular transport, cellular uptake, and others [23–25]. Here, we demonstrate three-dimensional high-resolution imaging of FNDs distributed in a macrophage cell by SAX microscopy. Figure 7 a) is an image of macrophages incubated with FNDs constructed by overlaying a confocal image of mitochondria and a SAX image of FNDs. To obtain the dual color image, we utilized the strong photostability of FNDs. Mitochondria was stained with Mitotracker Orange (Ex/Em: 554/576, Invitrogen), which has an emission wavelength that overlaps that from FNDs. We first observed FNDs and mitochondria simultaneously with a single PMT without SAX and obtained Fig. 7 b). After bleaching out the Mitotracker dye, the image of FNDs (Fig. 7 c)) was obtained with the same observation conditions as Fig. 7 b). The image of mitochondria (shown as green in Fig. 7 a)) was obtained by the subtraction of Fig. 7 c) from b). The FNDs were observed again by SAX microscopy with third harmonic demodulation, shown in Fig. 7 d), which is also seen in purple in Fig. 7 a). Comparing the confocal image (Fig. 7 c)) and SAX image (Fig. 7 d)) of FNDs, we confirmed the significant improvement of the spatial resolution in SAX microscopy, allowing more accurate

![Image](image_url)

Fig. 7. Fluorescence images of FNDs and mitochondria in macrophages in the focal plane (x-y image). a) Dual-color image constructed with a confocal image of mitochondria and a SAX image of FNDs, b) mitochondria and FNDs imaged simultaneously without SAX, c) confocal and d) SAX image of FNDs after bleaching the mitochondria staining dye. The distribution of mitochondria in a) was obtained by subtracting c) from b).
measurement of the distribution of FNDs after intracellular transport in the macrophage cell. In this experiment, the excitation laser and objective lens were same as in Fig. 5. The excitation intensity was modulated at 10 kHz and the intensities for confocal and SAX microscopy were 4 and 120 kW/cm$^2$, respectively. The pixel dwell time was 500 µs, and the image was composed of 480 × 480 pixels with a pixel size of 58 nm.

Figure 8 shows images of the FNDs and mitochondria in macrophages along the optical axis (x-z image), constructed using the same procedure as Fig. 7. Compared with focal plane (x-y) fluorescence images of FNDs obtained with confocal and SAX microscopy (Fig. 7), the x-z images shown in Fig. 8 also confirmed that SAX microscopy enhanced the spatial resolution along the optical axis as well as in the focal plane. In this experiment, the pixel number was 250 × 100, and other experimental conditions were the same as in Fig. 7.

These experiments demonstrate that the combination of SAX microscopy and FNDs is a powerful technique to image the distribution of particles taken into cells with high spatial resolution. Since FNDs are inert and nontoxic to living cells [15], the presented technique may be used for observation of localization or movement of particles associated with intracellular biological events.

8. Conclusion

In this paper, we reported the use of FNDs in SAX microscopy for high-resolution fluorescence imaging and dual-color imaging of biological samples. The high photostability of the FNDs allows us to observe a sample repeatedly with the spatial resolution beyond the diffraction limit. The scalability of the spatial resolution in SAX microscopy was also clearly demonstrated experimentally with harmonic demodulation at different frequencies. We also established the application of FNDs in imaging of biological samples, where the photostability of FNDs allows us to separate the fluorescence signals in the same wavelength range with only a single photodetector.

As indicated in the experimental results (Fig. 4), the practical resolution is limited by the signal-to-noise (SNR) ratio in the fluorescence detection. To obtain higher spatial resolution by demodulation of higher-order harmonic frequencies, further improvement of the SNR is required. The higher harmonic frequency components have around 10 to 1000 times lower signal intensity than the fundamental frequency component, as shown in Fig. 4. The high photostability is useful to increase the SNR, by allowing a longer exposure time. However, the temporal resolution of the imaging mode becomes lower. For improving the spatial resolution while keeping the temporal resolution, a FND possessing a larger absorption cross-section and a shorter fluorescence lifetime is desirable since it would allow rapid cycling of fluorescence.
excitation and emission, resulting an increase in the number of fluorescence photons detectable in a unit time. Even in such a case, a high excitation intensity is still required to image a sample with high spatial resolution, and, therefore, SAX microscopy with FNDs is most effectively applied to observations of fixed samples rather than live samples.

Acknowledgment

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