Demonstration of Super-Resolution Microscopy Using a High Numerical-Aperture Oil-Immersion Objective Lens

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We demonstrated super-resolution microscopy using a high numerical aperture (NA) oil immersion lens in acute optical alignment condition. In super-resolution microscopy, the pump beam is overlapped together with a doughnut-shaped erase beam in the focal plane, and fluorescence emission from the overlapping area is prevented. Alignment accuracy of these two beams determines the image properties of the microscope. Scanning fluorescence micro-beads in the focal plane, we confirmed the beam shape and its center position for the pump and erase beams respectively. Owing to the adjustment, we achieved a point-spread function with FWHM finer than 100 nm at least, which is predicted by the theory of super-resolution microscopy. [DOI: 10.1380/ejssnt.2008.175]

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I. INTRODUCTION

Super-resolution microscopy using fluorescence-depletion (FD) can give a spatial resolution finer than the diffraction limit [1, 2]. In FD, a first laser (pump beam) excites a molecule from the ground state \((S_0)\) to an \(S_1\) state, where the molecule emits fluorescence light. However, when a second laser (erase beam) further excites the molecule to an upper excited state \((S_n)\), the molecule returns from this state to the \(S_0\) state without any fluorescence emission through relaxation processes such as the inter-system crossing process [3, 4]. If the wavelength belongs to a fluorescence emission band, an induced-emission also contributes to preventing fluorescence emission [5]. As shown in Fig. 1(a), in super-resolution microscopy a Gaussian pump laser beam together with a doughnut beam, i.e., first-order Laguerre-Gaussian helical beam, is focused onto a sample [6]. The FD takes place in the overlapping area of the beams, and the fluorescence area disappears except at the immediate vicinity of the central point with the maximum fluorescence intensity. Thus, the fluorescence spot size is shrunk and becomes smaller than the diffraction limit, making this spot suitable as the scanning optical probe for super-resolution microscopy [7].

When an oil immersion lens with a high numerical aperture (NA) is used for super-resolution microscopy, the center hole of the erase spot has an FWHM of \(\sim 200\) nm, and the alignment of the pump and erase beams strongly affects the point-spread function (PSF). In order to theoretically investigate this effect for an oil immersion lens with an NA of 1.4, we calculated to the PSF based on the vectorial theory for FD and using the generalized vectorial Debye integral, assuming that a homogeneous thin fluorescence array contains randomly oriented Rhodamine-6G molecules [8, 9]. As shown in Fig. 1(a), the theoretical PSF for super-resolution microscopy has a Lorentzian shape, and its FWHM is expected to be 70 nm. However, the spatial resolution is restricted by the accuracy of the optical alignment in an actual experimental set-up. If the peak of the pump beam and the rim of the erase beam overlap due to misalignment, super-resolution cannot be achieved. For example, as shown in Fig. 1(b), when the erase and pump spots are misaligned by 160 nm, the profile of the fluorescence intensity is broadened and the peak intensity is significantly weakened. Thus, a decline of spatial resolution is caused together with a decrease of signal/noise ratio. To avoid this, the two beams should be aligned with an accuracy of the several tens of nanometers at least. Furthermore, when an oil immersion lens is adopted, oil must be inserted between the lens and a cover glass, and index matching is not perfect. Trivial wave aberration generated on the boundary between these materials distorts the shape of the beams. Therefore, by directly confirming their center position and the beam shape, we performed the optical alignment for achieving super-resolution microscopy. In this paper, we describe the alignment method and demonstrate super-resolution microscopy using a high NA oil immersion lens.

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FIG. 1: Theoretical PSF in super-resolution microscopy for a homogeneous thin layer of randomly oriented fluorescent Rhodamine-6G molecules. The PSF was calculated assuming a pump beam with a wavelength of 532 nm and an erase beam with a wavelength of 599 nm. For the calculation, the spectroscopic parameters for Rhodamine-6G listed in Ref. [9] and Table I are adopted. (a) The FWHM given by an oil immersion lens of 1.4 is 70 nm at a peak photon-flux of the erase beam of $2 \times 10^{25}$ photons/cm$^2$/sec in the focal plane. (b) The PSF of the fluorescence intensity is broadened and the peak intensity is significantly weakened, when the center points of the two beams are shifted by 160 nm with respect to each other.

II. EXPERIMENTAL SETUP AND ALIGNMENT METHOD

Figure 2 shows schematics of the experimental setup. In the experiment, the fluorescence spot profile was measured with and without erase-beam irradiation, i.e., super-resolution and normal measurements [10]. We demonstrated super-resolution microscopy using fluorescence micro-beads with Rhodamine-6G.

A. Experimental setup of laser scanning microscope

The pump and erase beams were generated by a Nd:YVO$_4$ solid-state-laser and a CW dye laser (Rhodamine 6G). We used the 532 nm second harmonic of the Nd:YVO$_4$ for the pump beam. The erase illumination with a wavelength of 599 nm was obtained from the dye laser excited by the Nd:YVO$_4$ laser. These beams were modulated to a light pulse with a width of 15 msec by two electro-optical modulators (EOM). Passing through a λ/4 plate and a spiral phase plate with 8 etched areas, the erase pulse beam was transformed into a circularly polarized first-order Laguerre-Gaussian helical beam by a spiral phase plate and a quarter-wave plate. Focusing both combined beams onto the sample on the stage by an oil immersion objective lens, the fluorescence signals were detected by a photo-multiplier tube (PMT). The signals summed at every observed pixel were stored in a personal computer.

To compare between the super-resolution measurement and the normal one under the same conditions, the system clock with the frequency of 200 kHz is used as the operation clock for the pump beam, and the frequency is divided by two for generating the operation clock for the erase beam. As shown in Fig. 3, the pump pulse is synchronized on every alternate pulse with the divided erase pulse. From these pulses, we generate two kinds of event pulses by a logic AND circuit, i.e., the pump pulse is irra-
the pump and erase beams are co-axially aligned or not. In the latter case, overlapping profile images of the pump and erase beams are obtained. Investigating the center position and shapes of these images, we can confirm whether the pump and erase beams are co-axially aligned or not.

In the alignment procedure, we adopt commercially used 100-nm-φ fluorescence micro-beads (Molecular Probes Co. Inc.: FluoSpheres). When the optical alignment procedure is performed, we use F8801 micro-beads (red bead) for the erase test beads, which have an absorption band from 570 nm to 610 nm and can emit fluorescence light when illuminated by either of the pump and the erase beams. Using F8801 micro-beads, the beam profiles of these beams can be measured. On the other hand, F8800 micro-beads (orange beads) with Rhodamine-6G were used for demonstrating super-resolution microscopy. They have an absorption band from 450 nm to 570 nm, and can be excited to the S<sub>1</sub> state only by the pump beam. Using the parameters of Table I, the erase beam can easily induce FD [13].

### Table I: Experimental parameters for super-resolution microscopy.

| Parameter                        | Value   |
|----------------------------------|---------|
| NA of objective lens             | 1.4     |
| FWHM of spot size of pump beam   | 220 nmφ @ λ = 532 nm |
| FWHM of hole size of erase beam  | 200 nmφ @ λ = 599 nm |
| Pump beam intensity              | 0.5 W   |
| Erase beam intensity             | 20 mW   |
| Repetition of pump beam pulse    | 200 KHz |
| Pulse width of pump beam         | 15 nsec |
| Pulse width of erase beam        | 20 nsec |

### C. Samples

We performed the optical alignment in super-resolution microscopy using an objective lens with NA=1.4 (Olympus Co. Ltd.: UPLSAPO 100XO). From Table I, the theoretical FWHM is 200 nm in the normal measurement. On the other hand, the FWHM in the super-resolution measurement is expected to be 70 nm.

Figure 4 shows the reflected light image of the pump and erase beams on the slide glass taken by the ICCD camera. From Fig. 4, it can be determined that the center of the reflected pump lights is in the central hole of the focused erase beam. This means that the two beams were pre-aligned co-axially. Next, we measured the shape of the focused erase beam by scanning an erase test beads in the focal plane. As shown in Fig. 5, the beam has fairly good cylindrical symmetry and near-zero intensity at the center, which is suitable for super-resolution microscopy. Finally, according to the logic sequence of Fig. 3, we observed the distance between the center positions of the focused pump and erase beams. Figure 6(a) shows a beads image with irradiating the only pump beam, i.e., the pump beam profile, and Fig. 6(b) is the profile with irradiating the pump and erase beam at the same time, i.e., the overlapped profile of these two beams. In order to loose the rim of the erase beam, we carefully adjusted intensities of these beams. We operated the pump and erase laser with 0.1 µW and 1 µW respectively. As shown in the cross-sectional profile (Fig. 6(c)), the peak position...
FIG. 4: Reflected light image of the pump and erase beams on the slide glass taken by the ICCD camera. The reflected pump light is focused in the center hole of the focused erase beam. Thus the pump and erase beams are pre-aligned co-axially.

of the pump beam was located within the center hole of the erase beam. Thus, we directly confirmed that the two beams were co-axially focused on the sample.

After the described alignment, we replaced the red fluorescence micro-bead with a slide glass with scattered orange beads, and compared the fluorescence spot images given by the super resolution measurement and the normal measurement. As shown in Fig. 7, the FWHM of 220 nm in the normal measurement (Fig. 7(a)) was shrunk to 110 nm in the super resolution measurement (Fig. 7(b)). When a Lorentzian function with an FWHM of 70 nm is adopted for the theoretical PSF for super-resolution microscopy, the convolution of the PSF with the spherical beads shape, i.e., beads image provides an FWHM of 110 nm. Thus, Fig. 7(b) indicates that the PSF given in this experiment should have the theoretical FWHM. We concluded that the pump and erase beams were aligned with enough accuracy to achieve super resolution and the obtained PSF has an FWHM smaller than the beads size of 100 nm at least.

FIG. 5: (a) Shape of the focused erase beam and (b) its cross-sectional profile measured by scanning an erase test bead in the focal plane. The beam has fairly good cylindrical symmetry and near-zero intensity at the center.

FIG. 6: Measured distance between the center positions of the focused pump and erase beams. (a) Beads image with irradiating the only pump beam, i.e., the pump beam profile. (b) Image with irradiating the pump and erase beam at the same time, i.e., the overlapped profile of these two beams. (c) Cross-sectional intensity distribution of the overlapped profile. The peak position of the pump beam was located within the center hole of the erase beam. Thus, we directly confirmed that the two beams were co-axially focused on the sample.

IV. DISCUSSION AND CONCLUSION

In this study, we adjusted the two x-y mirrors by hand for aligning the pump and erase beams. This method is very suitable in laboratory experimental setups with trial and error procedures. However, in commercial laser scanning microscopes, a simple and easy optical alignment is required [14]. For this purpose, an annular spiral phase plate technique may be used. In this technique, the erase beam, co-axially aligned with the pump beam, passes through the plate, and only the erase beam is deformed into a doughnut-shaped one. Let us assume that the pump and erase beams can be derived from the same output coupler of a single mode fiber, and collimated by
FIG. 7: Fluorescence spot images given by the normal measurement and super resolution measurement after alignment. The FWHM of (a) 220 nm in the normal measurement was shrunk to (b) 110 nm in the super resolution measurement. Considering the bead size, the FWHM of the PSF is estimated to be smaller than 100 nm.

the same apochromatic objective lens [15]. In such a system, the center position of the beams overlap completely, and one can achieve the optical alignment for super resolution microscopy within machine accuracy [16]. Based on the obtained results, we emphasize that the correct alignment could achieve the theoretical value of super-resolution in a commercial laser-scanning microscope.

We demonstrated super-resolution microscopy using a high NA oil immersion lens in an acute optical alignment condition. In this microscope system, the pump beam overlaps a doughnut-shaped erase beam in the focal plane, and fluorescence emission from an overlapping area is prevented. The image properties of the microscope depend crucially on the alignment accuracy of these two beams. Scanning fluorescence micro-beads in the focal plane, we confirmed the shape and center position of the pump and erase beams respectively. By adjusting the two beams, we achieved a PSF with an FWHM smaller than 100 nm at least, which is predicted by the theory of super-resolution microscopy.

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