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# Compact three-dimensional super-resolution system based on fluorescence emission difference microscopy

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A B S T R A C T

A compact microscope system for three-dimensional (3-D) super-resolution imaging is presented. The super-resolution capability of the system is based on a size-reduced effective 3-D point spread function generated through the fluorescence emission difference (FED) method. The appropriate polarization direction distribution and manipulation allows the panel active area of the spatial light modulator to be fully utilized. This allows simultaneous modulation of the incident light by two kinds of phase masks to be performed with a single spatial light modulator in order to generate a 3-D negative spot. The system is more compact than standard 3-D FED systems while maintaining all the advantages of 3-D FED microscopy. The experimental results demonstrated the improvement in 3-D resolution by nearly 1.7 times and 1.6 times compared to the classic confocal resolution in the lateral and axial directions, respectively.



## 1. Introduction

Since the concept of super-resolution was put forward, several such methods that break the classical diffraction barrier have been reported, many of which can even provide up to nanoscale optical resolution. High-resolution methods categorized on the basis of field of view, i.e., methods based on a classic confocal system, include stimulated emission depletion (STED) microscopy [1] and fluorescence emission difference (FED) microscopy [2]. Wide field methods that directly extract high-frequency information from the sample using a complex algorithm include stochastic optical reconstruction microscopy (STORM) [3], structured illumination microscopy (SIM) [4], and photo activated localization microscopy (PALM) [5,6]. In recent years, not only has the resolution of these methods been enhanced, but the imaging speed and system compactness have also improved. Furthermore, several of the methods mentioned above have been extended to three-dimensional (3-D) super-resolution [7–10] to reveal more spatial details. The fundamental features of ideal super-resolution microscopy include high resolution, fast imaging speed, 3-D imaging, and compact size. Considerable research has focused on these features, and super-resolution microscopy platforms have developed rapidly. For instance, as one of the earliest super-resolution methods, the comprehensive ability of STED microscopy has significantly improved since its inception, especially in terms of resolution and imaging speed. Hess et al. [6] and Harke et al. [7] reported 3-D STED imaging with a compact size microscope. The compact setup for 3-D imaging has gained attention recently, as it is economical and easy to install.

Here, FED microscopy is utilized to build an optical system that breaks the classical diffraction barrier. Three-dimensional FED [11,12] employs a spatial light modulator (SLM) to generate a negative focal spot. The negative spot can also be generated using a specific phase plate. One of the limitations of using a phase plate is that the phase retardation distribution is fixed. In contrast, the SLM is more flexible owing to the user-defined phase modulation pattern. Because of this, the usage of SLM is preferred, especially in the case where phase modulation of the incident light is required. Additionally, the similar utilization of SLM has been working well in STED systems [13–15]. Furthermore, based on SLM, one can apply adaptive optics and wavefront optimization to correct system aberrations [14,16,17]. In conventional 3-D FED microscopy, two additional wave plates are required to produce two spatial negative focal spots. For this purpose, the physical system needs two extra optical paths to avoid the replacement of wave plates for a different modulation beam. The advantage of the SLM is computer-controlled operation, through which different phase patterns can be

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assigned to produce two distinct negative focal spots. This operation not only eliminates the two wave plates from the system, but also restricts it to a single optical path. The advantage of the single optical beam is the optical alignment precision along the optical axis; namely, it would be significantly more difficult to accurately align multiple beams. This single optical beam results in a compact, easy to handle, and vibration-free system. In addition, the SLM is adaptive to a wide band of wavelengths, which would make this system applicable to multi-color imaging.

Regarding the reported 3-D FED system, one super-resolved image is retrieved from three images, i.e., a solid spot image and two negative spot images, which accordingly required three distinct illuminations of the sample [12]. This sequential illumination has two main drawbacks: sample drifting and reduced imaging speed. Sample drifting would result in a distorted image. An alternate strategy for compensating for these drawbacks in a 3-D FED setup is to use two SLMs. Under the simultaneous illumination of the sample with two SLMs, two negative spots excite the sample at the same time, improving the imaging speed by one third. However, this raises both the space and hardware costs.

The 3-D FED system reported in this paper is based on the utilization of a single SLM, and similar utilization in STED microscopy has been reported [15]. The three main advantages of the 3-D FED system presented here over those previously reported are its compact size, stability, and imaging speed. The exclusion of two wave plates or two SLMs reduces the system to a more compact form. The novel feature of this system is its stability against environmental factors due to its one-axis geometry. This system required only two sample scans to obtain one super-resolved image, thereby improving the imaging speed by one third.

## 2. Theory

In this method, the equation for the 3-D FED image is

$$I_{3DFED} = I_c - r\left(I_{xy} + I_z\right), \tag{1}$$

where $I_{3DFED}$ is the resultant 3-D FED image, $I_c$ is the normal confocal image using a solid spot scan, $I_{xy}$ and $I_z$ are the two negative spot images obtained by lateral and axial negative spot scanning, respectively, and $r$ is the subtraction factor for the two images. Further elaboration of the normal confocal image is given by

$$I_c = I_s \otimes \left[PSF_s \times \left(PSF_d \otimes P\right)\right], \tag{2}$$

where $I_s$ is the intensity distribution of the sample, $PSF_s$ and $PSF_d$ represent the excitation point spread function (PSF) and detection PSF, respectively, and P is the pinhole function. Thus, $PSF_s \times \left(PSF_d \otimes P\right)$ is the effective PSF of the normal confocal image. The mathematical representations of the two negative images, $I_{xy}$ and $I_z$, are

$$I_{xy} = I_s \otimes \left[PSF_{xy} \times \left(PSF_d \otimes P\right)\right], \tag{3}$$

$$I_z = I_s \otimes \left[PSF_z \times \left(PSF_d \otimes P\right)\right], \tag{4}$$

where $PSF_{xy}$ and $PSF_z$ are two negative excitation PSFs. By substituting Eqs. (2)–(4) into Eq. (1), the 3-D FED image is

$$I_{3DFED} = I_s \otimes \left\{\left[PSF_s - r\left(PSF_{xy} + PSF_z\right)\right] \times \left(PSF_d \otimes P\right)\right\}. \tag{5}$$

Thus, $\left[PSF_s - r\left(PSF_{xy} + PSF_z\right)\right] \times \left(PSF_d \otimes P\right)$ is the effective PSF of the 3-D FED image.

The two negative spots can be generated by phase modulation based on the vector diffraction model. Many systematic comparisons aimed at determining the optimum choice for confining the fluorescence spot in the lateral and axial directions using different devices have been reported [8,18,19]. An excitation beam modulated by a vortex 0–2$\pi$ phase mask yields a lateral negative focal spot, which has already been widely used in STED and FED microscopy. This negative focal spot is toroidal, being helpful for lateral resolution enhancement; however, it does not feature any excitation intensity in the axial direction, leaving the axial resolution unaffected. Therefore, in order to improve axial-direction resolution, a 0–$\pi$ phase mask is utilized to modulate the excitation beam to yield an axial direction negative focal spot. Fig. 1 shows the two patterns uploaded to the SLM and the phase retardation distribution. The phase retardation functions, $\Delta\alpha(\theta, \varphi)$, of the vortex 0–2$\pi$ mask and the 0–$\pi$ mask are respectively shown in Fig. 1(a) and (b), and their mathematical descriptions in polar coordinates [12] are respectively as follows:

$$\Delta\alpha_{xy}(\theta, \varphi) = \varphi, \varphi \in [0, 2\pi], \tag{6}$$

$$\Delta\alpha_z(\theta, \varphi) = \begin{cases} \pi, & \theta < \theta_{\max}/\sqrt{2}, \\ 0, & \theta \geq \theta_{\max}/\sqrt{2}. \end{cases} \tag{7}$$

Fig. 1(c) and (d) show the calculated spatial shape of the lateral and axial direction negative spots, and their $x$–$y$ and $x$–$z$ intensity distributions are shown in Fig. 1(e) and (f) and (g–h), respectively. From Fig. 1(e), it is clear that the $x$–$y$ intensity distribution of a modulated negative spot (0–$\pi$) is not zero; on the contrary, it gives a toroidal distribution with a larger shape. In theory, this spot has been utilized for lateral resolution enhancement. Because it is lower in intensity, this modulated negative spot (0–$\pi$) required higher excitation power illumination to improve the lateral resolution. Unfortunately, such modulation may not be suitable for 3-D resolution enhancement because the intensity before and after the focal plane will be much higher, leading to detector saturation and sample bleaching. The gray patterns uploaded to the SLM are also shown in Fig. 1(i) and (j). One can display blazed grating on the SLM to isolate modulated light from unmodulated light because unmodulated light may cause background noise in the final image. In this method, the modulation ratio of the SLM exceeds 93% and the background noise caused by unmodulated light is partly suppressed in the final image via the FED process. Therefore, we did not use blazed grating. The gray scale theoretically ranges from 0 to 255, yet experimentally, the value may deviate slightly from the theoretical value according to the SLM linearity.

## 3. Method

The system strategy, which is shown in Fig. 2(a), utilizes only one SLM to modulate the excitation beam. The output from a 50–60 Hz Ti : sapphire laser (Coherent Inc.; Santa Clara, CA) passes through an acousto-optical tunable filter (AOTF; AA Opto-electronic; Orsay, FRA). A single-mode fiber carries the beam light to a collimator (Thorlabs; Newton, NJ). The beam is converted to linearly polarized light by a polarizer (Thorlabs), and a half-wave plate (Thorlabs) is used to adjust the polarization direction. The linearly polarized and collimated beam is reflected through a D-shape mirror (Thorlabs) toward the left side of the SLM (POLUTO-NIR-011; HOLOEYE Photonics AG; Berlin, Germany). The pixel size of the SLM is 8 μm, and the active area is 1920 × 1080 pixels. The purpose of the D-shaped mirror is to reduce the angle of incidence for the SLM as much as possible, improving SLM performance.

In this single-SLM-based technique, the active area of the SLM has been divided into two equal parts from the centerline. The number of pixels in the right and left parts are the same. The two different phase patterns are assigned to each part, as shown in Fig. 2. Taking advantage of the SLM characteristic that only one direction of polarized light may be modulated, the polarization of the excitation beam is adjusted accordingly. The initial angle of the incident beam with polarization is defined as "$\beta$" in Fig. 2(b). According to the working principle of the setup, the excitation beam reflects off the left part of the SLM with modulation of only the p-component of polarization and travels toward a simple mirror (Edmund Optics; Barrington, NJ), as shown in Fig. 2(a). Following this trajectory, a lens and a quarter-wave plate (Union Optic; Wuhan, CHN) are mounted between the mirror and the SLM. The lens and the mirror collectively work as a 4f system to maintain the wavefront quality. On