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Saturated virtual fluorescence emission difference microscopy based on detector array

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ABSTRACT

Virtual fluorescence emission difference microscopy (vFED) has been proposed recently to enhance the lateral resolution of confocal microscopy with a detector array, implemented by scanning a doughnut-shaped pattern. Theoretically, the resolution can be enhanced by around 1.3-fold compared with that in confocal microscopy. For further improvement of the resolving ability of vFED, a novel method is presented utilizing fluorescence saturation for super-resolution imaging, which we called saturated virtual fluorescence emission difference microscopy (svFED). With a point detector array, matched solid and hollow point spread functions (PSF) can be obtained by photon reassignment, and the difference results between them can be used to boost the transverse resolution. Results show that the diffraction barrier can be surpassed by at least 34% compared with that in vFED and the resolution is around 2-fold higher than that in confocal microscopy.

1. Introduction

In the field of Fraunhofer diffraction (far-field) the resolving ability is limited instead of unrestricted shrinkage due to the existence of diffraction barrier. [1,2] Super-resolution fluorescence microscopy is a collection of microscopy techniques that are able to reach the resolution power beyond the classic diffraction limit. Since the emergence of these unprecedented inventions, high-resolution fluorescence microscopy has been applied to biological and biomedical research rapidly and widely. Confocal scanning microscopy, the most broadly used modern optical microscopy technique, can surpass the diffraction barrier by a factor of $\sqrt{2}$ [defined by the full width at half-maximum (FWHM) of the point spread function (PSF)]. [3] Besides, in the latest 20 years, several super-resolution methods have been proposed, such as stimulated emission depletion (STED) microscopy [4,5], structured illumination microscopy (SIM) [6], stochastic optical reconstruction microscopy (STORM) [7] and photo-activated localization microscopy (PALM) [8], all these methods have successfully broken the diffraction limits based on distinct physical processes. Fluorescence emission difference microscopy (FED) can be another method to improve the resolution implemented by the subtraction of two scanning images modulated by two different beams. [9–11] The sample is alternately scanned by a solid excitation pattern and a doughnut-shaped excitation pattern, and the results show that a sharper effective PSF could be created through subtraction and the resolution is enhanced by a factor of 2 at most. However, the outer contour of the doughnut-shaped excitation pattern, which is obtained by a circularly beam modulated by a vortex 0–2π phase plate, is much larger than the solid pattern, resulting in the existence of the negative pixel values. The negative pixel values would lead to information loss and image deformation.

Virtual fluorescence emission difference microscopy (vFED) [12] is recently proposed by using photon reassignment technique [13,14], which has been previously used in confocal microscopy to improve the signal noise ratio (SNR). In vFED, the sample is scanned only by a doughnut-shaped pattern and imaged by a detector array other than a single detector, which is much more convenient than the previous FED methods. After photon reassignment, all the digital processed hollow PSFs can be combined to be a solid pattern. Hence, the FED method can be used with a processed solid pattern and a hollow pattern, resulting in greatly simplified system and transverse resolution enhanced by at least 1.3-fold compared with that in confocal microscopy, or 1.8–2-fold higher than that in wide-field microscopy. However, even if the vFED proposed in Ma's [12] work improved the weight of high spatial frequency and the deformation is eliminated, the cut-off spatial frequency still remains the same. Therefore the transverse resolution is limited to improve further. The resolving ability which is determined by the FWHM of the hollow excitation

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pattern in vFED can be further improved if the narrower dark spot can be obtained, which can be solved by the introduce of the fluorescence saturation [15].

In this paper, the saturated fluorescence is considered and a method for super-resolution imaging that we called saturated virtual fluorescence emission difference microscopy (svFED) is presented to further improve the transverse resolution and suppress the deformation. Only one saturated hollow excitation pattern is used to illuminate the sample, which shows greatly simplified configuration and high imaging speed. Photon reassignment is also used in our method and the whole detector array is considered in this paper, the captured images scanned by the saturated hollow excitation pattern which could generate the nonlinear effect can reconstruct the image with the resolution enhanced by at least 34% compared with that in vFED method, and nearly 2-fold than that in the conventional confocal microscopy.

2. Theory

The schematic of the svFED system is shown in Fig. 1. After being collimated by the collimation lens, the excitation beam from laser becomes linearly polarized through the polarizer. The first quarter-wave plate helps to compensate the polarization ellipticity and the half-wave plate is used to adjust the linear polarization direction of the excitation beam, which will become circular polarization after passing through the last quarter-wave plate before the high numerical aperture (NA) oil-immersion objective and can be modulated to the doughnut-shaped spot by the 0–2π vortex phase plate. In the detection path, the fluorescence excited from the sample will be collected by the 15×15 detector array after passing through the band-pass filter and collection lens. To simplify the discussion, in the svFED system it can be assumed that the NA of the objectives equals to 1.4 and refractive index of the immersion oil is 1.518, regardless of the malposition and aberration of the objectives lens.

In svFED, a detector array replaces a single detector to collect the fluorescence signal from the sample and each point detector in the detector array is in the size of only 0.1 AU (1 AU equals to $1.22\lambda/2\mathrm{NA}$), which can be equivalent to an pinhole set to 0.1 AU. Hence similar to the confocal PSF, we can have the equation defining the effective PSF of the center point detector in the detector array,

$$h_c(x, y, z) = h_{exc}(x, y, z) * (h_{det}(x, y, z) \otimes a(x, y)) \quad (1)$$

where $a(x, y)$ is a function modeling the size of the center point detector, h_{exc} and h_{det} represent the excitation and detection PSF, respectively. According to the vectorial diffraction theory [16,17], the detection PSF can be defined as a Bessel function [18,19], and

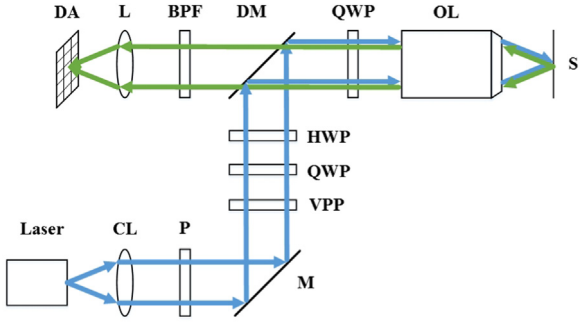

Fig. 1. Simplified schematic of the svFED setup. Abbreviations: CL, collimation lens; P, polarizer; M, mirror; VPP, 0–2π vortex phase plate; QWP, quarter-wave plate; HWP, half-wave plate; DM, dichroic mirror; OL, objective lens; S, sample; BPF, band-pass filter; L, collection lens; DA, 15×15 detector array.

the excitation PSF in svFED can be described as saturated hollow pattern

$$h_{exc}(x, y, z) = h_{hollow\_sat}(x, y, z) = \begin{cases} h_{hollow}(x, y, z)/\varsigma, & \text{where } h_{hollow}(x, y, z)/\varsigma \le h_{max} \\ h_{max}, & \text{where } h_{hollow}(x, y, z)/\varsigma > h_{max} \end{cases} \quad (2)$$

where $h_{sat\_hollow}(x, y, z)$ is the excitation PSF with the saturated hollow pattern, $h_{hollow}(x, y, z)$ represents the PSF of a general hollow pattern under non-saturated case, and ς is the saturation factor defined by $\varsigma = h_{th}/h_{max}$, where h_{th} is threshold value of the PSF and h_{max} is the peak value. Then, the effective PSF of the detector which is displaced in regard to the optical axis can be obtained based on the Eq. (1) can be shown as

$$h_d(x, y, z) = h_{exc}(x, y, z) * (h_{det}(x, y, z) \otimes a(x - d_x, y - d_y)) \quad (3)$$

where $a(x - d_x, y - d_y)$ represents the displaced point detector, d_x and d_y is the specific displacement in regard to the optical axis.

Obviously, except for the center detector, all the other detectors are not at the conjugated focal point resulting in the movement of the peak position of the detector PSF, so that the peak point of the effective PSF will shift [20]. The stimulation results of the PSFs of the center detector and two transversely displaced detectors of which d_x and d_y equal to 0.4 AU are shown in Fig. 2(a). When the excitation beam is saturated hollow pattern, the displacement of the detectors in the conjugated plane will result in the change of these effective PSF profiles. Although the zero points of the effective PSF keep on locating at the original point, the peak points are different, including the magnitude and the position. The photon reassignment process can be used by shifting the signal back to make the original point far from zero and summing up all the effective PSFs with a corresponding shift according to its position shown in Fig. 2(b). Hence the sum PSF shown in Fig. 2(c) can be obtained by

$$h_{pr}(x, y, z) = h_c(x, y, z) + \sum_{d_x, d_y} h_d(x + pd_x, y + pd_y, z) \quad (4)$$

where p is the shift factor to control the shift amount back to the original point. In Fig. 2(c), a solid

PSF can be achieved by the photon reassignment process from a hollow PSF, and have approximately same outer contour of the confocal PSF shown in Fig. 2(c) with a saturated hollow pattern excitation which can be shown as

$$h_{hollow\_con}(x, y, z) = h_{hollow\_sat}(x, y, z) * (h_{det}(x, y, z) \otimes \mathrm{pinhole}(x, y)) \quad (5)$$

where $\mathrm{pinhole}(x, y)$is the pinhole function of which the size is set to 0.6 AU in this paper.

In that the PSF is a solid pattern after the photon reassignment and the confocal PSF with saturated hollow excitation PSF is a hollow pattern, and these two PSFs have approximately same outer contours, the FED method can be used between them to obtain a better resolved reconstructed PSF,

$$h_{rec}(x, y, z) = h_{pr}(x, y, z) - q * h_{hollow\_sat}(x, y, z) \quad (6)$$

where q represents the subtractive factor. The reconstructed PSF shown in Fig. 2(c) can be used to reconstruct the sample's information. Fig. 2(d)–(f) show the magnitude distributions of the corresponding PSFs in Fig. 2(c) and the FED method can be obtained more intuitively.

Actually, considered the imaging process, the sample is not necessarily to be excited by the extra saturated hollow pattern in confocal condition. In svFED, sample need to be scanned by saturated hollow excitation pattern, and all detectors at different positions in the conjugated plane would collect the corresponding information. Therefore, the directly sum of the information, which