Enhancement of lateral resolution and optical sectioning capability of two-photon fluorescence microscopy by combining temporal-focusing with structured illumination

Keisuke Isobe,1,2,* Takanori Takeda,1,3 Kyoei Mochizuki,1,3 Qiyuan Song,2,4 Akira Suda,3 Fumihiko Kannari,4 Hiroyuki Kawano,3 Akiko Kumagai,5 Atsushi Miyawaki,2,5 and Katsumi Midorikawa1,2

1 Laser Technology Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
2 RIKEN Center for Advanced Photonics, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
3 Department of Physics, Graduate School of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
4 Department of Electronics and Electrical Engineering, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan
5 Laboratory for Cell Function Dynamics, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

* kisobe@riken.jp

Abstract: We demonstrate super-resolution imaging with background fluorescence rejection by interferometric temporal focusing microscopy, in which temporal focusing is combined with structured illumination. The lateral resolution and the optical sectioning capability are simultaneously improved by factors of 1.6 and 1.4, respectively, compared to conventional temporal focusing microscopy. Fluorescent beads (200 nm diameter) that are difficult to distinguish from the background fluorescence in conventional temporal focusing microscopy, are clearly visualized by interferometric temporal focusing microscopy.

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Multiphoton excited fluorescence (MPEF) microscopy has become a powerful tool for investigating biological phenomena because of its inherent advantages, including three-dimensional resolution without a confocal pinhole, high penetration depth with near-infrared light, and the ability to image structures with high resolution and contrast.

1. Introduction

Multiphoton excited fluorescence (MPEF) microscopy has become a powerful tool for investigating biological phenomena because of its inherent advantages, including three-dimensional resolution without a confocal pinhole, high penetration depth with near-infrared light, and the ability to image structures with high resolution and contrast.
light excitation, and reduced out-of-focus photon-induced damage and photobleaching [1–3].

Especially, MPEF microscopy benefits from the ability to image deeper within samples than confocal fluorescence microscopy, when near-IR excitation in the range for maximum optical transparency in biological systems is employed [4–8]. Nonetheless, deep imaging is intrinsically difficult because the excitation light is attenuated by scattering and absorption in the sample. To maintain sufficient intensity at a focus significantly deep in the scattering media, the energy of the excitation pulse must be increased. A regenerative amplifier producing 150-fs pulses at the μJ level has been used to image green-fluorescent-protein-labeled neurons at a depth of up to 1 mm within the neocortex [4]. However, increasing the pulse energy results in an increase of background fluorescence, which includes two-photon excited fluorescence (TPEF) signals generated in out-of-focus regions. This background fluorescence limits the maximum imaging depth [5]. Indeed, extensive efforts have been made to overcome this limitation [6–16]. The decreased scattering of excitation light in the sample, which has been achieved by using TPEF at 1280 nm [6, 7] and three-photon excited fluorescence at 1700 nm [8], has extended the maximum imaging depth. Temporal focusing (TF) suppresses out-of-focus signals in wide-field TPEF microscopy [9, 10]. TF microscopy combined with two-color two-photon microscopy, which spatially separates the two excitation wavelengths such that no signal corresponding to the sum-frequency generation can be generated in out-of-focus regions, allows the enhancement of the optical sectioning capability [11]. Adaptive optics can be used to reject the background by differential aberration imaging [12] and to recover diffraction-limited performance by compensating for wavefront distortion of the excitation pulse [13] in TPEF microscopy. The out-of-focus background in various point-scanning nonlinear microscopies has been rejected by spatial overlap modulation of two-color pulses, which creates spatial nonlinear intensity modulation near the focal point [14, 15]. The suppression of the background fluorescence in TPEF microscopy has been also achieved by utilizing photoactivatable fluorophores, which remain in a non-fluorescent state until optically triggered [16]. This technique is similar to cyclic-sequential-multiphoton excitation microscopy using reversible photoswitchable fluorophores [17]. In addition, structured illumination microscopy [18] and focal modulation microscopy [19], which employ spatial intensity modulation near the focal point, have been used to reject the out-of-focus background in one-photon excited fluorescence microscopy.

In a wide-field MPEF microscope, it is necessary to use a two-dimensional (2D) detector such as a charged coupled device (CCD) camera and a complementary metal oxide semiconductor (CMOS) camera [20–26]. Then, the fluorescence photons generated in a 2D spatial matrix within the sample must be imaged to their conjugate positions on the 2D detector. However, if the emitted photons are scattered within the sample, the scattered photons, which are not correctly mapped to the conjugate image position, turn into the background fluorescence in the image [26]. The scattered background fluorescence has been suppressed by introducing a segmented detector such as a multi-anode photomultiplier tube and utilizing descanned detection [26]. This configuration allows the multifocal microscope to operate in a similar fashion to that of a point-scanning microscope. However, this technique cannot be applied to wide-field MPEF microscopy using TF due to the lack of space between foci. On the other hand, TF microscopy has the greatest potential to increase the frame rate because there is no need for beam scanning. TPEF video-rate imaging over large areas of 5000-20000 μm² has been achieved by TF microscopy using amplified pulses [27, 28]. Recently, the HiLo microscopy technique, which generates an optically sectioned image by post-processing the uniformly illuminated image and the structured light illuminated image [29], has been used to reject the scattered background fluorescence and the out-of-focus background fluorescence in TF microscopy [30]. The structured illumination technique can also achieve super-resolution in wide-field one-photon fluorescence imaging [31, 32]. Through illumination with periodic patterns, high spatial frequency sample information is down-converted to a lower frequency, which can be supported by the optical transfer function (OTF) of a typical microscope. Shifting the down-converted components back to their true position in Fourier space extends the OTF of the microscope, yielding a super-resolution.
image. However, the enhancement of the lateral resolution by the structured illumination technique combined with wide-field MPEF microscopy has not yet been demonstrated experimentally.

In this paper, we demonstrate super-resolution imaging with background fluorescence suppression by interferometric TF (ITF) microscopy in which the structured illumination technique is combined with TF microscopy. The lateral resolution and the optical sectioning capability of TPEF imaging can be simultaneously enhanced by ITF microscopy. In ITF microscopy, periodic illumination patterns are generated near the focal plane by spatial interference of two TF pulses, and fluorescence is generated with two-photon excitation by the TF pulses. Choi et al. used only the fundamental spatial-frequency component of the periodic pattern to reject the background fluorescence in TF microscopy [30]. However, the second harmonic spatial-frequency component of the periodic pattern is also generated because the TPEF intensity is proportional to the square of the excitation intensity. Thus, we employ not only the fundamental spatial-frequency component of the periodic pattern but also the second harmonic spatial-frequency component, which is composed of the down-converted components. TPEF images reconstructed by shifting the down-converted components back to their true position can provide remarkable super-resolution and background rejection.

2. Interferometric temporal focusing microscopy

The basic principle of the ITF technique is illustrated in Fig. 1. To generate TF pulses, two spectrally dispersed pulses are focused into a sample by an objective lens and all the spectral components in each pulse are recombined only at the focal plane of the objective lens. Thus, pulse duration becomes a function of the distance from the focal plane, with the shortest pulse duration occurring at the focal plane [9, 10]. Therefore, the out-of-focus TPEF can be suppressed because of the TF effect. Furthermore, the two spatially divided pulses are not overlapped in space at the back aperture of the objective lens, but are overlapped only near the focal plane. Thus, spatial interference patterns produced by the two TF pulses appear only near the focal plane. The ballistic fluorescence light emitted with the two-photon excitation of the interfering light with periodic patterns allows the patterns to be projected onto a 2D detector. However, the fluorescence light scattered within the sample does not correctly produce the periodic patterns on the 2D detector. The out-of-focus fluorescence image also does not contain the periodic patterns because the out-of-focus background fluorescence is produced by one of the two TF pulses, rather than both simultaneously. Consequently, by extracting the periodic patterns from the measured TPEF images, not only the scattered background fluorescence but also the out-of-focus background fluorescence can be rejected. The concept for rejecting the out-of-focus background fluorescence in ITF microscopy is similar to that in TF microscopy combined with two-color two-photon microscopy [11].
In wide-field fluorescence microscopy, the measured image can be described as a convolution of the emitted fluorescence with the point spread function (PSF) of the incoherent imaging system, \( h(r) \). To avoid degradation of spatial resolution by fluorescence saturation, TPEF microscopy is generally used under the condition that the fluorescence intensity is proportional to the square of the excitation intensity. In this situation, the TPEF image of fluorescence samples acquired by an excitation light with a periodic pattern can be expressed as

\[
S_n(r) = \left\{ C(r) I_{\text{ex}}^2(r, \phi_n) \right\} \otimes h(r),
\]

where \( C(r) \) is the local fluorophore concentration and \( \otimes \) denotes convolution. Here the square of the excitation intensity, \( I_{\text{ex}}^2(r, \phi_n) \) is given by

\[
I_{\text{ex}}^2(r, \phi_n) = \left[ I_0(r) \{1 + \alpha \cos(k_0 y + \phi_0)\} \right]^2
= I_0^2(r) \left[ 1 + \frac{\alpha^2}{2} + \alpha \left( e^{i(k_0 y + \phi_0)} + e^{-i(k_0 y + \phi_0)} \right) \right.
+ \left. \frac{\alpha^2}{4} \left( e^{2i(k_0 y + \phi_0)} + e^{-2i(k_0 y + \phi_0)} \right) \right],
\]

where \( I_0(r) \) is the time-averaged intensity, \( \alpha \) denotes a modulation depth, and \( k_0 \) and \( \phi_0 \) are the fundamental spatial frequency and phase shift of the periodic pattern, respectively. As shown in Eq. (2), five spatial frequency components, \( -2k_0, -k_0, 0, +k_0, \) and \( +2k_0 \), are included in the periodic pattern. To extract each periodic pattern, we take five images, \( S_m(r) \) at the phase shifts, \( \phi_m = \phi_0 + m \phi_s \), from \( m = 0 \) to 4. Here the phase step, \( \phi_s \) is \( 2\pi/5 \), and \( \phi_0 \) is the offset phase. The \( l \)th order spatial frequency component, \( F_l(r) \) from \( l = -2 \) to 2, which is located around at the \( l \)th order spatial frequency \( l k_0 \), can be extracted by using the principle of homodyne detection:

\[
F_l(r) = \sum_{m=0}^{4} S_m(r) e^{inkle} \propto \left\{ C(r) I_{\text{ex}}^2(r) e^{i(lk_0 y + \phi_l)} \right\} \otimes h(r) = \left\{ D(r) e^{i(lk_0 y + \phi_l)} \right\} \otimes h(r),
\]
where \( D(\mathbf{r}) = C(\mathbf{r})F_{\lambda}^2(\mathbf{r}) \). Because the scattered background and the out-of-focus background do not produce the periodic pattern, they are contained in the 0th order spatial frequency (DC) component, \( F_0(\mathbf{r}) \). Hence, in order to suppress the background fluorescence, the final image is reconstructed from \( F_2(\mathbf{r}), F_1(\mathbf{r}), F_1(\mathbf{r}), \) and \( F_2(\mathbf{r}) \) only. The periodic pattern can be removed by the following equation:

\[
F_{\text{ITF}}(\mathbf{r}) = \left\{ \left[ F_1(\mathbf{r}) \right]^2 + \left| F_2(\mathbf{r}) \right|^2 \right\}^{1/2} = \left\{ F_1(\mathbf{r})F_1(\mathbf{r}) + F_2(\mathbf{r})F_2(\mathbf{r}) \right\}^{1/2}.
\]

(4)

Alternatively, \( F_{\text{ITF}}(\mathbf{r}) \) can be formed as

\[
F_{\text{ITF}}(\mathbf{r}) = \left\{ \frac{4}{2} \left( S_0 - S_m \right)^2 + \frac{4}{2} \left( S_1 - S_m \right)^2 + \frac{4}{2} \left( S_2 - S_m \right)^2 + \left( S_3 - S_4 \right)^2 \right\}^{1/2}.
\]

(5)

We call the image reconstructed by Eq. (4) or (5) the non-super-resolution ITF (NSR-ITF) image.

In order to obtain a super-resolution image, the offset phase \( \phi_0 \) and the \( l \)th order spatial frequency \( k_0 \) are employed. In Fourier space, Eq. (3) can be written as

\[
\hat{F}_j(\mathbf{k}) \propto \hat{D}(\mathbf{k} - /k_0)\hat{h}(\mathbf{k})e^{i\phi_0},
\]

(6)

where \( k_0 = (0, k_0) \). The Fourier spectrum can be extended by shifting the \( l \)th spatial frequency component \( \hat{F}_j(\mathbf{k}) \) to \( \hat{F}_j(\mathbf{k} + 1k_0) \) and combining the shifted frequency components:

\[
\hat{F}_{\text{SR-I} \text{TF}}(\mathbf{k}) = \sum_{l=2}^{5} a_l \hat{F}_j(\mathbf{k} + 1k_0) e^{-i\phi_0} = \hat{D}(\mathbf{k}) \sum_{l=2}^{5} b_l \hat{h}(\mathbf{k} + 1k_0),
\]

(7)

where \( a_l \) and \( b_l \) are the weighting factors. The super-resolution image can be acquired by the inverse Fourier transform of Eq. (7):

\[
F_{\text{SR-I} \text{TF}}(\mathbf{r}) = \sum_{l=2}^{5} a_l F_l(\mathbf{r}) e^{-i\phi_l + \phi_0}.
\]

(8)

In ITF microscopy, the cut-off spatial frequency of the excitation OTF, whose excitation beam passes through the edge of the back aperture of the objective lens, cannot be used as the pattern frequency. If the cut-off spatial frequency of the excitation OTF is selected as the pattern frequency, the spectral components except for the central wavelength are blocked at the back aperture of the objective lens. Thus, the medium frequency of the excitation OTF is employed as the pattern frequency. Therefore, even if the DC component \( F_0(\mathbf{r}) \) is removed, the OTF reconstructed by Eq. (7) doesn’t exhibit defects around a spatial frequency of 0, which is compensated for by the other components \( F_{l\neq0}(\mathbf{r}) \). Consequently, in order to reject the background fluorescence, \( a_0 \) in Eq. (8) can be set to 0. We call the image reconstructed by Eq. (8) the super-resolution ITF (SR-ITF) image.

In order to acquire the fundamental spatial frequency \( k_0 \) from the measured images \( S_m(\mathbf{r}) \), we calculate the cross-correlation between \( \hat{F}_j(\mathbf{k}) \) and \( \hat{F}_j(\mathbf{k}) \) :

\[
XC_l(\mathbf{k}) = \left| \iint \hat{F}_j(\mathbf{k}^1)\hat{F}_j(\mathbf{k}^1 - \mathbf{k})d\mathbf{k}^1 \right|.
\]

(9)

The cross-correlation between \( \hat{F}_j(\mathbf{k}) \) and \( \hat{F}_j(\mathbf{k}) \) should be high at \( k_0 \). Thus, as \( k_0 \), we use the spatial frequency for maximizing the cross-correlation between \( \hat{F}_j(\mathbf{k}) \) and \( \hat{F}_j(\mathbf{k}) \). The offset phase \( \phi_0 \) is also determined from the measured images \( S_m(\mathbf{r}) \) by calculating the cross-correlation:
where \( \text{Re}\{F\} \) denotes the real part of the complex \( F \). If the offset phase is correct, the cross-correlation \( XC_{\phi}(r) \) should be high at \( r = 0 \). Thus, we find the offset phase that maximizes \( XC_{\phi}(0) \).

Figure 2 shows the flowchart of the algorithm for reconstructing the SR-ITF image from the measured images. First, \( F_{1}(r) \) is calculated from the measured images \( S_{m}(r) \). Next, \( XC_{\phi}(k) \) is acquired from the fast Fourier transform (FFT) of \( F_{1}(r)F_{2}(r) \). Then, the spatial frequency for maximizing \( XC_{\phi}(k) \) is employed as \( k_{0} \). And then, \( F_{1}(r)e^{-i(k_{0}r+\phi_{0})} \) is calculated from \( F_{1}(r)e^{-i\phi_{0}} \) and \( \phi_{0} \). Finally, the SR-ITF image is reconstructed from a linear combination of \( F_{1}(r)e^{-i(k_{0}r+\phi_{0})} \). The data processing time was 2 seconds per SR-ITF image (64-bit OS, Intel Core i7 CPU at 3.50 GHz, and 16 GB RAM).

\[
XC_{\phi}(r) = \int \text{Re}\{F_{1}(r)e^{-i(k_{0}r+\phi_{0})}\}F_{1}^{*}(r-r)dr,
\]

3. Experimental

3.1 Experimental setup

The experimental setup is illustrated in Fig. 3. As an excitation light source, we used an optical parametric oscillator (OPO; Coherent, Mira-OPO-IR-FAN) operating at a central wavelength of 1100 nm and a repetition rate of 76 MHz, which was pumped by a mode-locked Ti:sapphire oscillator (Coherent, Mira-900F) at 830 nm. To compensate for dispersion of all the optical components, the OPO pulses were passed through an S-TIH6 prism pair. The output from the prism pair was divided into two beams by a reflective beam splitter [33] composed of a pair of silver coated mirrors. One of these mirrors was fixed on a mirror holder and the other was mounted on a piezoelectric transducer stage (nPoint, NPX25A) for applying...
the phase shift. The interferometry using the reflective beam splitter was stable enough to allow precise measurement of interference fringes in an attosecond pulse train [34]. The two beams were diffracted by the reflective diffraction grating with a groove density of 830 grooves/mm. To avoid the tilt of the TF plane on the sample, the incident angle on the grating was adjusted so that the diffraction angle of the central wavelength was zero. The two spectrally dispersed pulses were focused into the samples by a water immersion objective lens (OB; Olympus, UPLSAP60 × W, NA 1.2). Because the laser power was not high enough to achieve wide-field TPEF imaging at deeper penetration depths, the excitation intensity at the focal plane of the objective lens was increased by using line-focusing along the y direction. The two line-focused beams were interfered only at the focal plane of the objective lens. At the focal plane, the pulse duration was 142 fs. The generated fluorescence signal was collected by the objective lens and separated from the excitation pulses using a dichroic mirror (DM; Thorlabs, DMLP900). The focal plane was imaged onto a CMOS camera (Hamamatsu Photonics, ORCA-Flash2.8) and TPEF images were acquired with an exposure time of 50 ms. To exclude the residual excitation pulses, a short-pass filter (SPF, Semrock, FF01-890/SP-25) was used and a band-pass filter (BPF; Semrock, FF01-550/88-25) was employed to select the detection wavelength. By scanning the sample in the lateral (y) and axial (z) directions with stepping motor-driven stages, the image was obtained. To apply the phase shift, the piezoelectric transducer stage was moved with a step size of 110 nm and a settling time of 4 ms per step. It took 270 ms to acquire the five images at different phase shifts.

![Fig. 3. ITF microscope setup. OPO: optical parametric oscillator, PS: periscope to rotate the spatial beam profile, CCL: concave cylindrical lens, DM: dichroic mirror, OB: objective lens, SPF: short-pass filter, BPF: band-pass filter.](image)

**3.2 Sample preparation**

To demonstrate the enhancement of the optical sectioning capability of ITF microscopy, we prepared a solution of Rhodamine B with a concentration of 100 μM, which was set in a glass container with an optical path length of 70 μm. To investigate the suppression of the scattered background fluorescence and the resolution enhancement by ITF microscopy, we prepared tissue-like phantoms, which contain nile red (535/575) fluorescent polystyrene beads with a diameter of 2 μm (Molecular Probes F8825) or orange (540/560) fluorescent polystyrene beads with a diameter of 200 nm (Molecular Probes F8809). We added 40 μL of the 2-μm fluorescent bead solution with a concentration of \(4.55 \times 10^9\) beads/mL and 40 μL of the 200-nm fluorescent bead solution with a concentration of \(4.55 \times 10^{12}\) beads/mL to two different 160-μL quantities of low-melting-point agarose gel, then pipetted the gels onto 35-mm Petri dishes with cover glass bottoms, and covered them with an additional cover glass. We also prepared another tissue-like phantom, which contained both the 2-μm fluorescent beads and 200-nm fluorescent beads.
3.3 Results

We measured the axial (z) response in order to demonstrate the enhancement of the optical sectioning capability of ITF microscopy. Figure 4(a) shows the measured one-dimensional (1D) signal distribution along the axial direction near the interface between the cover glass and the Rhodamine B solution. The input power was 8 mW. The fundamental spatial frequency of the periodic pattern was 5.4 rad/μm. The axial response of the NSR-ITF microscope is steeper than that of the TF microscope. The axial response of the SR-ITF microscope composed of ± k₀ and ± 2k₀ components (a₂ = a₁ = a₀ = a₁ = a₂ = 1, a₀ = 0) is comparable to that of the NSR-ITF microscope, while the axial response of the SR-ITF microscope composed of all frequency components (a₂ = a₁ = a₀ = a₁ = a₂ = 1) is comparable to that of the TF microscope. The full width at half maximums (FWHMs) of the first derivatives of the 1D signal distributions along the axial direction for the NSR-ITF and TF microscopes were 0.89 μm and 1.27 μm, respectively. This result indicates that the optical sectioning capability of the NSR-ITF microscope is 1.4 times higher than that of the TF microscope. Thus, ITF microscopy without the DC component allows the enhancement of the optical sectioning capability.

We obtained TPEF images of an orange fluorescent bead with a diameter of 200 nm to determine the spatial resolution of ITF microscopy. The input power was 10 mW. Figure 4(b) shows the measured signal distribution along the lateral (y) direction. FWHMs for TF microscopy, NSR-ITF microscopy and SR-ITF microscopy composed of the all frequency components, ± k₀ and ± 2k₀ components (a₂ = a₁ = a₀ = a₁ = a₂ = 1, a₀ = 0), and ± 2k₀ components (a₂ = a₁ = 1, a₁ = a₀ = a₁ = 0) were 0.29 μm, 0.29 μm, 0.26 μm, 0.24 μm, and 0.19 μm, respectively. We calculated FWHMs of effective PSFs by using the following equation: Δ = √(Δ₁² - Δ₂²), where Δ₁ is the FWHM of the measured bead image and Δ₂ is the FWHM of a model Gaussian function of the bead. Lateral FWHMs of effective PSFs for TF microscopy, NSR-ITF microscopy, SR-ITF microscopies composed of the all frequency components, ± k₀ and ± 2k₀ components, and ± 2k₀ components were estimated to be 0.28 μm, 0.28 μm, 0.24 μm, 0.22 μm, and 0.17 μm, respectively. The lateral resolution has been improved by a factor of 1.6. It should be noted that the lateral resolution and the optical sectioning capability can be simultaneously enhanced by SR-ITF microscopy without the DC component.
We acquired TPEF images of fluorescent beads in the tissue-like phantom in order to demonstrate the suppression of the scattered background fluorescence by ITF microscopy. The phantom contained fluorescent polystyrene beads with a diameter of 2 μm at a concentration of 9.0 × 10^8 beads/mL, which corresponds to a scattering coefficient of 78 cm⁻¹ for 575 nm, as scatterers and tracers. Figure 5 shows the TPEF images in the xy plane at various penetration depths. The TPEF image at each penetration depth was reconstructed from 40 ITF images obtained by scanning the sample with a step size of 250 nm in the y direction. It took 15 seconds to acquire the TPEF image at each penetration depth by using ITF microscopy. In order to compensate the loss of excitation power at the focal plane, the input powers at penetration depths of 5 μm, 55 μm, 155 μm and 255 μm were set to 1.6 mW, 4.1 mW, 41 mW and 163 mW, respectively. However, because the laser power was not high enough to compensate the loss of excitation power at a penetration depth of 255 μm, the exposure time was increased to 100 ms, which corresponds to the image acquisition time of 25 seconds. As the penetration depth increased, the scattered background fluorescence extending around the beads increases in the images obtained by TF microscopy and SR-ITF microscopy composed of the all frequency components (a₂ = a₁ = a₀ = 0, a₁ = a₂ = 1).
contrast, the scattered background fluorescence could be rejected by NSR-ITF microscopy and SR-ITF microscopy composed of $\pm k_0$ and $\pm 2k_0$ components ($a_2 = a_3 = a_1 = a_2 = 1$, $a_0 = 0$) only. Therefore, ITF microscopy without the DC component allows the rejection of the scattered background fluorescence.

Fig. 5. TPEF images of the fluorescent beads obtained by (left) TF microscopy, (the second from the left) NSR-ITF microscopy, and SR-ITF microscopies composed of (the third from the left) the all frequency components and (right) $\pm k_0$ and $\pm 2k_0$ components only.

We applied ITF microscopy to TPEF imaging of fluorescent beads in the tissue-like phantom, which contained fluorescent polystyrene beads with a diameter of 200 nm at a concentration of $9.0 \times 10^{11}$ beads/mL. The input power was 10 mW at the penetration depth of 5 $\mu$m, and was increased by a factor of $\exp(az)$ with increasing penetration depth. $a$ was set to 0.002 $\mu$m$^{-1}$. Figure 6 shows the images in the $yz$ plane measured by NSR-ITF microscopy, and SR-ITF microscopies composed of $\pm k_0$ and $\pm 2k_0$ components ($a_2 = a_3 = a_1 = a_2 = 1$, $a_0 = 0$), 25% $\pm k_0$ and $\pm 2k_0$ components ($a_1 = a_1 = 0.25$, $a_2 = a_2 = 1$, $a_0 = 0$), and $\pm 2k_0$ components ($a_1 = a_0 = a_1 = 0$, $a_2 = a_2 = 1$). The TPEF image was reconstructed from 2000 ITF images obtained at depth increments of 100 nm. The total image acquisition time was 680 seconds. As shown in Fig. 7, the improved lateral resolution of SR-ITF microscopy results in better spatial separation of the beads, and the spatial separation of the beads is enhanced as the contribution of $\pm 2k_0$ components increases. This is because the high spatial frequency components are enhanced. However, the signal-to-noise ratio decreases as the contribution of $\pm 2k_0$ components increases because the signal intensities of $\pm 2k_0$ components are at least 4 times lower than those of $\pm k_0$ components.
Fig. 6. TPEF images of the 200-nm fluorescent beads obtained by (left) NSR-ITF microscopy, and SR-ITF microscopies composed of (the second from the left) $\pm k_0$ and $\pm 2k_0$ components, (the third from the left) 25% $\pm k_0$ and $\pm 2k_0$ components, and (right) $\pm 2k_0$ components.
Fig. 7. Normalized signal profiles of NSR-ITF microscopy (red), and SR-ITF microscopies composed of ± $k_0$ and ± 2$k_0$ components (green), 25% ± $k_0$ and ± 2$k_0$ components (orange), and ± 2$k_0$ components (pink) along the lateral direction indicated by (a) blue, (b) green, and (c) red arrows in Fig. 6.

We used ITF microscopy to image fluorescent beads in the tissue-like phantom, which contained fluorescent polystyrene beads with a diameter of 200 nm at a concentration of 9.0 × $10^{11}$ beads/mL, as well as beads with a diameter of 2 μm at a concentration of 9.0 × $10^8$ beads/mL. The input power was 68 mW. The observed images at penetration depths of 140 μm and 141 μm, which were normalized at each penetration depth, are depicted in Fig. 8(a). The TPEF image at each penetration depth was reconstructed from 28 ITF images obtained by scanning the sample with a step size of 250 nm in the $y$ direction. It took 10 seconds to...
acquire the TPEF image at each penetration depth by employing ITF microscopy. The TPEF signals from the 200-nm beads are difficult to distinguish from the out-of-focus background fluorescence and the scattered background fluorescence in the images obtained by TF microscopy and SR-ITF microscopy composed of the all frequency components. In contrast, the 200-nm beads are clearly visualized in the images acquired by NSR-ITF microscopy and SR-ITF microscopy composed of ± \( k_0 \) and ± 2\( k_0 \) components. The out-of-focus background signals derive from the TPEF signals of the 2-μm beads located at different penetration depths. Thus, the TPEF signals of the 2-μm beads are spread beyond 2 μm. The left-hand and right-hand 2-μm fluorescent beads in the images were observed at penetration depths of about 143 μm and 142 μm, respectively. Because the optical sectioning capabilities of NSR-ITF microscopy and SR-ITF microscopy without the DC component is higher than those of TF microscopy and SR-ITF microscopy with the DC component, the TPEF signal from the 2-μm beads located at different penetration depths could be suppressed by NSR-ITF and SR-ITF without the DC component. In addition, the fluorescence light from the 2-μm beads, which is scattered within the sample, becomes large background for the signals of the 200-nm beads because the TPEF intensity from the 2-μm beads is much higher than that from the 200-nm beads. Since NSR-ITF and SR-ITF without the DC component allows the rejection of the scattered background fluorescence, the scattered background fluorescence from the 2-μm beads could be eliminated.

4. Discussion and conclusions

We have demonstrated that ITF microscopy combining TF with structured illumination makes it possible to enhance the lateral resolution and the optical sectioning capability by factors of 1.6 and 1.4, respectively, and to reject both the out-of-focus background fluorescence and the scattered background fluorescence. In these demonstrations, line-focusing was employed because the laser power was not high enough to achieve wide-field imaging. By using amplified pulses at the μJ level, our technique can be easily applied to wide-field imaging. In addition, the amplified pulses allow the use of TPEF saturation, which enables us to further improve the spatial resolution. By using saturation nonlinearity in one-photon excited fluorescence, the spatial resolution has been further enhanced [35–38]. However, in one-photon excitation, fluorophores located at out-of-focus regions can be quickly photobleached due to the high excitation intensity of the saturation. This problem may be solved by two-photon excitation based on the TF technique.

Since the used camera has a 12-bit digitizer, the dynamic range of the detection system was 4096. In the images shown in Fig. 8, the effective dynamic range was dramatically narrowed to 400 by the large background fluorescence. In addition, the signal intensities of ± 2\( k_0 \) components are at least 4 times lower than those of DC and ± \( k_0 \) components. Thus, the effective dynamic range for ± 2\( k_0 \) components was further narrowed to 25. In contrast, the noise level composed of the shot noise for the dark current, the readout noise and the thermal noise was 10. Therefore, the signal-to-noise ratio for the ± 2\( k_0 \) components was low. By using a camera with a high dynamic range, the signal-to-noise ratio for the ± 2\( k_0 \) components could be improved.

In order to achieve an isotropic enhancement in lateral resolution by the structured illumination technique, periodic illumination patterns must be rotated several times [31]. The periodic illumination patterns at various orientations can be generated by rotating the excitation grid [31] or by using a programmable spatial light modulator [39, 40]. In ITF microscopy, the dispersive system for generating TF pulses must be also rotated simultaneously. However, an isotropic enhancement in lateral resolution by ITF microscopy might be achievable by generating the two spatially divided TF pulses using a single programmable spatial light modulator.
Fig. 8. (a) TPEF images of the fluorescent beads obtained by TF microscopy, NSR microscopy, and SR-ITF microscopies composed of the all frequency components, and ± k₀ and ± 2k₀. (b-e) Normalized signal profiles of TF microscopy (black), NSR-ITF microscopy (red), and SR-ITF microscopies composed of the all frequency components (blue), and ± k₀ and ± 2k₀ components (green) along the lateral (x and y) direction indicated by (b) blue, (c) green, (d) pink, and (e) red arrows in (a).

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