Interferometric temporal focusing microscopy using three-photon excitation fluorescence

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Abstract: Super-resolution microscopy has become a powerful tool for biological research. However, its spatial resolution and imaging depth are limited, largely due to background light. Interferometric temporal focusing (ITF) microscopy, which combines structured illumination microscopy and three-photon excitation fluorescence microscopy, can overcome these limitations. Here, we demonstrate ITF microscopy using three-photon excitation fluorescence, which has a spatial resolution of 106 nm at an imaging depth of 100 µm with an excitation wavelength of 1060 nm.

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1. Introduction

Various types of fluorescence microscopies, such as super-resolution microscopy and multiphoton excitation fluorescence microscopy have become powerful tools for investigating biological phenomena. However, with these techniques, there are trade-offs among the spatial resolution, penetration depth, temporal resolution, and imaging area. For example, although super-resolution optical microscopy is capable of achieving high spatial resolution beyond the diffraction limit [1–9], the penetration depth is much lower than those of confocal microscopy and multiphoton microscopy. Recently, to boost the usability of these microscopies, the drawbacks of each have been compensated by combining different techniques. Super-resolution techniques combined with two-photon excitation fluorescence (2PEF) microscopy have led to super-resolution deep imaging [10–16]. Stimulated emission depletion (STED) microscopy [2], which employs a tightly focused laser as an excitation light source, has been combined with laser scanning 2PEF microscopy [17,18] to provide super-resolution deep imaging [10–12]. On the other hand, structured illumination microscopy (SIM) [7–9] has been coupled with 2PEF temporal focusing (TF) microscopy [16], which allows depth-resolved wide-field two-photon imaging without laser scanning [19,20].
Despite these efforts, further enhancement of the spatial resolution and penetration depth of large-area imaging is required to visualize complex biological phenomena, such as brain function, which is related to interactions between neurons, and the regulation of neuronal and spine structure. Here we demonstrate a more advanced deep-imaging technique with high spatio-temporal resolution as well as large-area imaging capability. In our scheme, SIM is combined with TF microscopy using three-photon excitation fluorescence (3PEF) with near-infrared pump light [21].

The enhancement of spatial resolution results in a reduction in signal intensity because the signal volume decreases. A small signal can easily be buried in background fluorescence generated in out-of-focus regions and/or by scattered photons within the sample. In our scheme, the out-of-focus background can be suppressed by using 3PEF microscopy, in which the excitation area is more localized near the focus of the excitation light [22,23] than that in 2PEF microscopy [17,18]. The drawback of the spatial resolution of multi-photon excitation fluorescence microscopy being lower than that of super-resolution microscopy employing visible light due to the use of near-infrared light is overcome by employing a combination of SIM and three-photon excitation. The use of near-infrared light at 1060 nm as the pump is indispensable for deep imaging since it can penetrate deeply into biological samples. Consequently, the spatial resolution of 3PEF-ITF microscopy using 1060-nm pulses can be enhanced up to 106 nm, which corresponds to one-tenth of the pump wavelength at a penetration depth of 100 µm. In addition, the temporal focusing technique employed in our scheme allows large-area (5000–20000 µm²) video-rate 2PEF imaging [24,25] while maintaining the spatio-temporal resolution.

In section 2, we introduce the principle of 3PEF-ITF microscopy and describe the experimental setup. In section 3, we report the enhancement of the optical sectioning capability and lateral resolution, and demonstrate the application of 3PEF imaging to mouse brain tissue. In section 4, we summarize the paper.

2. Methods

2.1 Theory

The principle of 3PEF-ITF microscopy can be derived from that of TF microscopy [26]. In TF microscopy, by recombining all the spectral components in a spectrally dispersed pulse only at the focal plane of an objective lens, the pulse duration becomes the function of the distance from the focal plane [19,20]. Consequently, the out-of-focus 3PEF can be suppressed even if wide-field illumination is employed. The axial response of TF microscopy using 3PEF is given by [21]

$$A_{TF}(z) = \frac{1}{1 + \left(\frac{z}{z_R}\right)^2}. \quad (1)$$

Here, $z_R$ is related to the optical sectioning capability by the TF effect, which depends on the numerical aperture. A two-dimensional (2D) image is recorded by a 2D detector, which is placed at the conjugate position of the focal plane. The detected image $D(x, y, z)$ can be expressed as

$$D(x, y, z) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} S(x', y', z') I^3(x', y', z-z') \times A_{TF}(z-z') h(x-x', y-y', z-z') dx' dy' dz', \quad (2)$$

where $S(x, y, z)$ is the density distribution of the fluorophore in the sample, $I_0(x, y, z)$ is the time-averaged intensity distribution of a TF pulse, and $h(x, y, z)$ is the point spread function (PSF) of the detection system. Here, a three-dimensional (3D) image is obtained by scanning the sample along the axial direction. If we assume that periodic illumination patterns are
generated by spatial interference of three TF pulses, the time-averaged electric field of the excitation light is expressed as [26]

\[ E_{\text{av}}(x, y, z) = E_0 \left[ e^{j k_0 x} + \alpha e^{j(k_0 \sin \theta + z \cos \theta) + \phi_0} + \alpha e^{j(-k_0 \sin \theta + z \cos \theta) + \phi_0} \right] , \]  

where \( k_0 \) is the wavevector of the central wavelength, \( \alpha \) is the modulation depth, \( \theta \) is the angle between the central and outer beams, and \( \phi_0 \) is the phase shift of the periodic pattern. The cube of the time-averaged intensity can be written as

\[ I_{\text{av}}^3(x, y, z) = \left| E_0 \right|^2 \sum_{j=-6}^{6} A_j^{(3)}(z) e^{j(k_{j,0} z \cos \theta)} , \]  

where

\[ A_j^{(3)}(z) = 1 + 3 \left\{ 7 + 4 \cos(2k_{j,0} z) \right\} \alpha^2 + 3 \left\{ 11 + 8 \cos(2k_{j,0} z) \right\} \alpha^4 + \alpha^6 \]  

\[ A_{j1}^{(3)}(z) = 6 \cos(k_{j,0} z) \alpha + 2 \left\{ 15 \cos(k_{j,0} z) + \cos(3k_{j,0} z) \right\} \alpha^2 + 30 \cos(k_{j,0} z) \alpha^4 \]  

\[ A_{j2}^{(3)}(z) = 3 + 2 \cos(2k_{j,0} z) \right\} \alpha^2 + 6 \left\{ 4 + 3 \cos(2k_{j,0} z) \right\} \alpha^4 + 6\alpha^6 \]  

\[ A_{j3}^{(3)}(z) = 2 \left\{ 9 \cos(k_{j,0} z) + \cos(3k_{j,0} z) \right\} \alpha^3 + 18 \cos(k_{j,0} z) \alpha^5 \]  

\[ A_{j4}^{(3)}(z) = 3 \left\{ 3 + 2 \cos(2k_{j,0} z) \right\} \alpha^4 + 3\alpha^6 \]  

\[ A_{j5}^{(3)}(z) = 6 \cos(k_{j,0} z) \alpha^5 \]  

\[ A_{j6}^{(3)}(z) = \alpha^6 \]  

\[ k_{j,0} = k_0 \sin \theta \]  

\[ k_{j,0} = k_0 \left( 1 - \cos \theta \right) . \]  

Using Eqs. (2) and (4), the detected image can be rewritten as

\[ D_n(x, y, z) = \left| E_0 \right|^2 \sum_{j=-6}^{6} S(x', y', z') e^{j(k_{j,0} z \cos \theta)} \otimes \left[ A_{TF}^{(3)}(z) A_j^{(3)}(z) h(x, y, z) \right] \]  

where \( \otimes \) denotes a convolution. The detected image in Fourier space is given by

\[ \tilde{D}_n(k_x, k_y, k_z) = \left| E_0 \right|^2 \sum_{j=-6}^{6} \tilde{S}(k_x, k_y, jk_{j,0}, k_z) e^{j \phi_0 \times \left[ \tilde{A}_{TF}(k_z) \otimes \tilde{A}_j^{(3)}(k_z) \otimes \tilde{h}(k_z, k_y, k_z) \right] } \]  

\[ = \sum_{j=-6}^{6} \tilde{F}_j(k_x, k_y, k_z) e^{j \phi_0 \times \left[ \tilde{A}_{TF}(k_z) \otimes \tilde{A}_j^{(3)}(k_z) \otimes \tilde{h}(k_z, k_y, k_z) \right] } \]  

where

\[ \tilde{F}_j(k_x, k_y, k_z) = \left| E_0 \right|^2 \sum_{j=-6}^{6} \tilde{S}(k_x, k_y, jk_{j,0}, k_z) e^{j \phi_0 \times \left[ \tilde{A}_{TF}(k_z) \otimes \tilde{A}_j^{(3)}(k_z) \otimes \tilde{h}(k_z, k_y, k_z) \right] } . \]  

The detected image includes 13 Fourier components, \( \tilde{F}_j(k_x, k_y, k_z) \), at spatial frequencies of around \( jk_{j,0} \) (\( j = -6, -5, \ldots, 0, \ldots, 5, 6 \)). As shown in Eq. (7), high-spatial-frequency sample information is down-converted to lower-frequency information in the \( k_z \) direction, which can be supported by the optical transfer function (OTF) of the diffraction-limited system. To
extend the OTF by shifting the down-converted components back to their true positions in Fourier space, each Fourier component must be separated by using the principle of homodyne detection:

\[ H_j(k_x, k_y, k_z) = \frac{1}{13} \sum_{m=0}^{12} D_m(k_x, k_y, k_z) e^{-i m \phi}, \]

where 13 images are obtained with a phase step \( \phi_s \) of \( \frac{2\pi}{13} \). Here the phase shifts are given by \( \phi_m = \phi_{\text{offset}} + m \phi_s \), where \( m = 0 \) to 12, and \( \phi_{\text{offset}} \) is the offset phase. After shifting the separated Fourier components back to their true positions, the offset phase is determined by the phase difference between \( H_2(k_x, k_y - 2k_{\phi_0}, z) \) and \( H_1(k_x, k_y - k_{\phi_0}, z) \) in the overlap region at each axial position \( z \) and is corrected. Recombining the shifted Fourier components extends the cut-off spatial frequencies in the \( k_y \) and \( k_z \) directions to \( k_{yd} + 6k_{\phi_0} \) and \( k_{zd} + k_{TF} + 3k_{\phi_0} \), respectively. Here, \( k_{yd} \) and \( k_{zd} \) are the cut-off spatial frequencies of the detection system OTF in the \( k_y \) and \( k_z \) directions, and \( k_{TF} \) is the cut-off spatial frequency of the TF effect. To enhance the 3D spatial resolution, the orientation of the periodic pattern must be rotated in the lateral direction. The background fluorescence can be rejected by ignoring the Fourier component at the spatial frequency of around 0, \( H_0 \), because it does not produce a periodic pattern on the 2D detector \[16\].

2.2 Experimental setup

Figure 1 shows a schematic diagram of the 3PEF-ITF microscopy setup. As an excitation light source, we utilized a in-house-developed Yb-fiber chirped pulse amplifier (CPA) system, which produces 92-fs 9.0-µJ 1060-nm pulses at a repetition rate of 200 kHz \[21\]. Output pulses from the Yb-fiber CPA system were input into a digital micromirror device (DMD: DLP4500NIR, Texas Instruments) with an incident angle of 23.1° \[26\]. Periodic patterns applied on the DMD generated 0th and ± 1st order diffracted light as three TF pulses and were imaged at the focal plane of a water-immersion objective lens with a numerical aperture (NA) of 1.2 (OB; UPLSAPO 60 × W, Olympus). The imaging area was 30 × 30 µm². The spatial frequencies of the generated periodic patterns were 4.2 rad/µm. The generated fluorescence signal was collected by the same objective lens and separated from the excitation pulses by a dichroic mirror (DM; DMLP900, Thorlabs). To remove the residual excitation pulses, a short-pass filter (SPF, FF01-890/SP-25, Semrock) and a band-pass filter (BPF; FF02-435/40-25 for fluorescent beads, FF01-405/150-25 for DAPI, and FF01-550/88-25 for SYTO83, Semrock) were used. The fluorescence images were amplified using an image intensifier (II: C9546, Hamamatsu Photonics) and recorded by a CMOS camera (ORCA-Flash4.0, Hamamatsu Photonics). The 3D image was obtained by scanning the sample in the axial direction with a stepping motor-driven sample stage. The signal-to-noise ratios (SNRs) of TF images and each Fourier component to reconstruct ITF images were improved by filtering out the high-frequency noise in the axial direction, which had a frequency higher than the cut-off spatial frequency of the OTF in the axial direction for the diffraction-limited detection system. Because the signal intensities of the separated Fourier components were different, their combining ratio was determined so that their signal intensities were equal to each other.
3. Results and discussion

3.1 Optical sectioning capability

To estimate the optical sectioning capability of 3PEF-ITF microscopy, we measured the signal distribution from one layer of 100-nm fluorescent beads (F8797, Molecular Probes) along the axial direction. The input power was 69 mW and the exposure time was 50 ms. The emission wavelength of the beads was 440 nm. Figure 2 shows the signal distributions acquired using 3PEF-TF and 3PEF-ITF microscopies. The full width at half maximum (FWHM) was 2.60 µm for 3PEF-TF microscopy and 0.86 µm for 3PEF-ITF microscopy. The optical sectioning capability of 3PEF-ITF microscopy was enhanced three-fold compared to that of 3PEF-TF microscopy. It was also confirmed that the optical sectioning capability of 3PEF-ITF microscopy was 0.75 times the wavelength of the excitation light (1060 nm).

3.2 Lateral resolution

The lateral resolution of 3PEF-ITF microscopy was estimated from the lateral signal distribution of 100-nm fluorescent beads (F8797, Molecular Probes), which were mixed in agarose gel. Figure 3(a) shows the one-dimensional signal distributions of a bead along the lateral direction, which were acquired with 3PEF-TF and 3PEF-ITF microscopies at a depth of 100 µm. The input power was 138 mW and the exposure time was 200 ms. Since the SNR
of the sixth harmonic Fourier component \( H_{±6} \) was too low to be used in the reconstruction of the ITF image, the ITF image was reconstructed from \( H_{±1}, H_{±2}, H_{±3}, H_{±4}, \) and \( H_{±5}. \) By fitting each distribution with Gaussian functions, the FWHMs in the lateral direction were evaluated to be 255 nm for 3PEF-TF and 106 nm for 3PEF-ITF microscopy. The sample condition was similar to that in [26], where the spatial resolutions of 2PEF-TF and 2PEF-ITF microscopies were independent of the penetration depth because the wavefront was not disturbed. Thus, the spatial resolution should be independent of the penetration depth. By averaging the FWHMs obtained from seven images of an isolated bead located in the depth range from 50 µm to 100 µm, we estimated the lateral resolutions of 3PEF-TF and 3PEF-ITF microscopies to be 295 nm and 130 nm, respectively. As shown in Figs. 3(b), (c), and (d), it is difficult to identify the gap between neighboring beads using 3PEF-TF, whereas it is clearly visualized due to the improved resolution by using 3PEF-ITF microscopy. For the diffraction-limited detection system at a wavelength of 440 nm and an NA of 1.2, the cut-off spatial frequency \( k_{yd} \) of the OTF in the lateral direction was theoretically calculated to be 34.3 rad/µm, which resulted in the 183-nm FWHM of the PSF. As the fundamental spatial frequency \( k_{y0} \) of the periodic pattern in the lateral direction was 4.2 rad/µm, the cut-off spatial frequency of the effective OTF in the lateral direction was theoretically extended to 59.5 rad/µm \( (= k_{yd} + 6k_{y0}) \), which corresponds to the 105-nm FWHM of the PSF. In practice, the sixth harmonic Fourier component could not be employed due to the low SNR. Thus, the cut-off spatial frequency of the extended OTF experimentally decreased to 55.3 rad/µm \( (= k_{yd} + 5k_{y0}) \), which resulted in the 113-nm FWHM of the PSF. However, the maximum order of the harmonic Fourier components could expand up to the fifth harmonic due to the suppression of the out-of-focus background fluorescence with three-photon excitation [21], while those in 2PEF-ITF microscopy corresponded to the third harmonic [26]. Compared with the theoretical FWHM at the diffraction limit, the FWHM measured by 3PEF-TF microscopy is slightly broadened due to the wavefront distortion in the detection system, whereas the FWHM measured by 3PEF-ITF microscopy is close to the theoretical FWHM. This is because ITF microscopy has a resistance to the resolution degradation due to wavefront distortion [27]. Since the wavefront distortion becomes larger in practical tissue imaging, the resolution would be degraded. In that case, adaptive optics, which is a technique for correcting the wavefront distortion caused by the inhomogeneous refractive index distribution of biological tissue [28], is useful.

Fig. 3. (a) Signal distributions of a fluorescent bead along the lateral direction, acquired using 3PEF-TF and 3PEF-ITF microscopies. (b, c) Cross-sectional images of 100-nm fluorescent beads acquired using (b) 3PEF-TF and (c) 3PEF-ITF microscopies, respectively. (d) Signal distributions along the yellow solid lines shown in panels (b) and (c). Scale bars in (b) and (c) both correspond to 1 µm.
3.3 Observation using a biological sample

Finally, we applied 3PEF-ITF microscopy to observe fixed mouse brain tissue. The brain was fixed with 4% paraformaldehyde (PFA). A coronal section of the cerebral cortex with a thickness of 100 µm was prepared and nuclei were stained with DAPI and SYTO83, which bind to RNA as well as DNA. The emission wavelengths of DAPI and SYTO83 are 461 nm and 559 nm, respectively. The fluorescence images of DAPI-stained nuclei and SYTO83-stained nuclei were acquired by two-photon excitation and three-photon excitation using a single wavelength of 1060 nm. The input powers were 275 mW for 3PEF and 83 mW for 2PEF. The exposure time was 30 ms. The full results for the z-stack of 3PEF and 2PEF images acquired with 3PEF-TF, 3PEF-ITF, 2PEF-TF and 2PEF-ITF microscopies can be seen in an online movie (Visualization 1) provided in the supplementary material. The movie was reconstructed from 226 xy images obtained at depth increments of 200 nm. Figures 4(a), 4(b), 4(c), and 4(d) show single frames from Visualization 1 for 3PEF-TF, 3PEF-ITF, 2PEF-TF, and 2PEF-ITF microscopies, respectively. The out-of-focus background fluorescence was larger than that in deep imaging of the 100-nm fluorescent beads because the densities of the DAPI and SYTO83 were higher than that of the fluorescent beads. Thus, the maximum orders of the harmonic Fourier components in 3PEF-ITF and 2PEF-ITF microscopies were limited to fourth and second harmonics, respectively. Since fluorescence from DAPI-RNA is much weaker than from DAPI-DNA [29], the 3PEF images cannot be compared with 2PEF images precisely. However, we could confirm that the out-of-focus background fluorescence for 3PEF-TF microscopy was lower than that for 2PEF-TF microscopy. Thus, the maximum order of the harmonic Fourier components in 3PEF-ITF microscopy was larger than that in 2PEF-ITF microscopy. Consequently, 3PEF-ITF microscopy is more suitable for enhancing the spatial resolution in deep imaging than 2PEF-ITF microscopy. In addition, we also found that a combination of 2PEF-ITF and 3PEF-ITF microscopies could enable dual-color deep imaging because the background fluorescence could be rejected by ignoring the DC component \((H_0\) in Eq. (9)) after the data acquisition. As shown in Figs. 4(e), (f), and (g), the gap is clearly recognized for 3PEF-ITF microscopy compared with 3PEF-TF microscopy because of the enhancement in the optical sectioning capability and the background fluorescence rejection. A comparison of Figs. 4(h) and (i) also demonstrates the lateral resolution enhancement. A thin fiber-like structure was visualized only using 3PEF-ITF microscopy, and as shown in Fig. 4(j), the size of the structure was less than 200 nm. Figures 4(k) and (l) also show single frames from Visualization 1 for 3PEF-TF and 3PEF-ITF microscopies, respectively. The 3PEF-ITF images clearly show the high chromatin density at the nuclear envelope due to the improved resolution compared with the 3PEF-TF images. This result is similar to the comparison of the distributions of chromatin in cultured cells using single molecule localization microscopy (SMLM) [4–6] and wide-field fluorescence microscopy [30]. Nevertheless, the imaging depth of 3PEF-ITF microscopy is greater than that of SMLM. For the diffraction-limited detection systems at wavelengths of 461 nm for DAPI and 559 nm for SYTO83 and an NA of 1.2, the cut-off spatial frequencies \(k_{cut}\) were theoretically calculated to be 32.7 rad/µm and 27.0 rad/µm, respectively. Thus, the cut-off spatial frequencies of the extended OTF of 3PEF-ITF and 2PEF-ITF microscopies should be extended to 49.5 rad/µm \((= k_{cut} + 4k_0)\) and 35.2 rad/µm \((= k_{cut} + 2k_0)\), respectively. Finally, we discuss the photo-damage during imaging. The threshold average power for damage caused by absorption of water in the living brain is 500 mW [31]. Thus, the average power used in this experiment is set less than the threshold average power for damage. The threshold fluence for ablative damage of biological tissues ranges from 1.5 to 2.2 J/cm² in the wavelength region of 800–1450 nm [32]. Since the laser fluence in this experiment was 0.15 J/cm², which was estimated from the pulse energy of 1.38 µJ/pulse and the illumination area of 30 × 30 µm², ablative damage could be neglected.
Fig. 4. (a-d) 3PEF and 2PEF images of a fixed mouse brain stained with DAPI and SYTO83, acquired using (a) 3PEF-TF, (b) 3PEF-ITF, (c) 2PEF-TF, and (d) 2PEF-ITF microscopies. (e, f) Magnified views of the red squares indicated in (e) panel a and (f) panel b. (h, i) Magnified views of yellow squares in (h) panel a and (i) panel b. (g, j) Signal distributions of the dashed lines in (g) panels e and f, and in (i) panels h and i, respectively. (k, l) 3PEF images of a fixed mouse brain stained with DAPI, obtained using (k) 3PEF-TF and (l) 3PEF-ITF microscopies. (a-d) and (k, l) were acquired at depths of 20 µm and 18 µm from the position where the fluorescence signal was detected for the first time, respectively.
4. Conclusion

We have developed 3PEF-ITF microscopy by combining 3PEF-TF microscopy with SIM. The lateral resolution and optical sectioning capability were 106 nm and 860 nm, respectively, which corresponded to respective enhancements of 2.3 and 3.0 times compared to 3PEF-TF microscopy. The spatial resolution of 106 nm could be achieved even with 1060-nm excitation at a penetration depth of approximately 100 μm. In the future, 3PEF-ITF microscopy could be a powerful tool for investigating biological phenomena related to, for example, brain function. In the field of neuroscience, the super-resolution deep imaging technique demonstrated here could be applied to observe losses of spines and alterations in synaptic plasticity. Extending the field-of-view of 3PEF-ITF microscopy could easily be achieved by increasing the laser power, yielding visualization of communication between neurons. Higher power excitation might induce thermal damage on the sample, but this could be reduced by decreasing the repetition rate of the excitation laser. Even lower repetition rates can provide video-rate imaging to observe the propagation of action potential for each neuron because laser scanning is not required in the TF technique. In addition, decreasing the repetition rate to less than 1 MHz can suppress photobleaching of fluorescent dyes via excited-state absorption from the triplet state.

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