Wavefront sensorless adaptive optics temporal focusing-based multiphoton microscopy

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Abstract: Temporal profile distortions reduce excitation efficiency and image quality in temporal focusing-based multiphoton microscopy. In order to compensate the distortions, a wavefront sensorless adaptive optics system (AOS) was integrated into the microscope. The feedback control signal of the AOS was acquired from local image intensity maximization via a hill-climbing algorithm. The control signal was then utilized to drive a deformable mirror in such a way as to eliminate the distortions. With the AOS correction, not only is the axial excitation symmetrically refocused, but the axial resolution with full two-photon excited fluorescence (TPEF) intensity is also maintained. Hence, the contrast of the TPEF image of a R6G-doped PMMA thin film is enhanced along with a 3.7-fold increase in intensity. Furthermore, the TPEF image quality of 1μm fluorescent beads sealed in agarose gel at different depths is improved.

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OCIS codes: (110.1080) Active or adaptive optics; (170.3880) Medical and biological imaging; (180.4315) Nonlinear microscopy.

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Multiphoton excited fluorescence microscopy has been widely utilized for biological imaging since 1990 [1]. With superior features such as minimum invasiveness, lower photobleaching, and deeper penetration depth, the microscopy is particularly suitable for imaging thick tissues and living animals [2]. Furthermore, second harmonic generation (SHG), another phenomenon of nonlinear optics, can be effectively utilized to acquire information of non-

1. Introduction

Multiphoton excited fluorescence microscopy has been widely utilized for biological imaging since 1990 [1]. With superior features such as minimum invasiveness, lower photobleaching, and deeper penetration depth, the microscopy is particularly suitable for imaging thick tissues and living animals [2]. Furthermore, second harmonic generation (SHG), another phenomenon of nonlinear optics, can be effectively utilized to acquire information of non-

#209713 - $15.00 USD
Received 7 Apr 2014; revised 2 May 2014; accepted 5 May 2014; published 9 May 2014
(C) 2014 OSA 1 June 2014 | Vol. 5, No. 6 | DOI:10.1364/BOE.5.001768 | BIOMEDICAL OPTICS EXPRESS 1769
centrosymmetry directly within specimens, such as collagen within tissues [3], without fluorescent labeling. Because of the nature of nonlinear excitation, both two-photon excited fluorescence (TPEF) and SHG are usually excited by employing a high numerical aperture (NA) objective lens and an ultrafast laser for spatially and temporally generating an extremely high electromagnetic field. TPEF and SHG only occur in a small region near the focal point of the objective lens, and hence high spatial resolution for three-dimensional (3D) imaging of specimens can be obtained via pixel by pixel point scanning. In order to increase the speed of multiphoton imaging, recent studies have shown that temporal focusing technique can provide widefield and axially-resolved multiphoton imaging [4–6]. Only in the focal plane do the different frequency components overlap in phase, and produce a short, high-peak power pulse, allowing effective multiphoton excitation to occur. Compared to conventional beam scanning multiphoton microscopy, widefield multiphoton microscopy using the temporal focusing technique detects the overall fluorescence and harmonic generation signal of the entire illumination area, which depends on the laser beam spot size and the magnification of the microscope. The advantage of widefield multiphoton microscopy is that less time is required to capture one frame, enabling a fast frame rate for capturing dynamic events [7,8] or fast microfabrication applications [9,10]. With a fast, high-sensitivity camera and an ultrahigh peak power laser, an imaging rate of a few hundred frames per second can be achieved in widefield multiphoton microscopy [11].

Optical distortions in multiphoton microscopes and wavefront distortions caused by turbid biospecimens often degrade excitation efficiency and image quality, especially for deep tissue imaging. To overcome these shortcomings, an adaptive optics system (AOS) is implemented [12–14]. AOS incorporates a wavefront corrector, such as a deformable mirror (DM) or a spatial light modulator (SLM), driven by an active control loop using feedback signals from a wavefront sensor (WFS) to compensate the optical distortions and wavefront distortions [15–18]. AOS has been used to improve the image resolution and contrast for pixel by pixel point-scanning multiphoton microscopy by enhancing the quality of the focus [16,19]. However, if a real-time AOS is incorporated into a point-scanning multiphoton microscope, the overall frame rate could be further reduced due to the extra wavefront sensing, computation, and correction by the AOS; therefore, such a system is more suitable for time-independent applications [20,21]. In contrast, fast imaging acquisition of a temporal focusing-based multiphoton microscopy may alleviate the speed-constraint of the AOS, potentially enabling real-time applications. Distortions in the temporal focusing-based multiphoton microscopy or in specimens distort temporal profiles of the excitation pulse at the focal plane of the objective lens [22], leading to reductions in axial resolution, image intensity, and contrast quality. Conventional widefield AO microscopy could correct depth aberrations by controlling the DM according to the path length difference induced by different refractive indexes [23]. Unpredictable aberrations in the specimens could be analyzed as Zernike polynomials from the reference guide-star and be corrected [24]. Regarding the complexity of spatial and temporal distortions in widefield temporal focusing-based multiphoton microscopy, this study focused on the temporal distortion compensation that restores the excitation pulse width at the focal plane to achieve better widefield optical sectioning. Research is being conducted into the shaping of ultrashort pulses to compensate distortions for optimized spatiotemporal focusing and thereby demonstrate focal spot enhancement [25,26].

A wavefront sensor in AOS, such as the Shack-Hartman wavefront sensor (SHWS), is widely used to measure and analyze wavefront distortion. However, it is difficult to straightforwardly measure the wavefront in specimens and obtain the necessary information for correction. In this regard, coherence-gated wavefront sensing with interferometry [15] and reference guide-star with SHWS [24,27,28] have been adopted for wavefront sensing. On the other hand, wavefront sensorless AOSs have been adopted to compensate aberrations by maximizing image quality based on specific definitions, e.g. mean intensity [17,29–31]. In
order to accelerate the control loop in a wavefront sensorless AOS, an appropriate approach for the element selection of a wavefront corrector, such as a specific individual element [29], low-order Zernike modes [30], or different segments of SLM [17] should be chosen. The sensorless AOS is capable of computing the feedback control signals to drive the wavefront corrector via a suitable algorithm to maximize the image quality value, which also serves as an indication of the aberration compensation level [31,32]. In this study, a wavefront sensorless image-based AOS has been integrated into a temporal focusing-based multiphoton microscope for axially-resolved optical imaging. The AOS uses a 32-element DM as the wavefront corrector, and employs a hill-climbing algorithm to compute an appropriate control signal to drive the DM such that the effects of optical aberrations and specimen-induced temporal distortions are reduced. Experimental results show that the temporal focusing-based multiphoton microscope with the wavefront sensorless AOS not only recovers the axial resolution but also refocuses the axial excitation. In addition, the TPEF intensity of an R6G-doped PMMA thin film is increased by 3.7-fold. Contrast enhancements of 1μm fluorescent beads fixed in agarose gel at different sectioning depths can clearly be also observed.

2. Optical setup and principle

2.1. Overall system

Figure 1 shows the temporal focusing-based multiphoton microscope with a wavefront sensorless image-based AOS. The laser source of the widefield multiphoton microscope is a Ti:sapphire ultrafast amplifier (Spitfire Pro., Newport, USA) coupled with a Ti:sapphire ultrafast oscillator (Tsunami, Spectra-Physics, USA) as the seed beam of the amplifier. The ultrafast amplifier has a peak power of 400 μJ/pulse, a pulse width of 90 fs, and a repetition rate of 10 kHz, which is sufficient to excite TPEF with an excitation area larger than 100 × 100 μm². The center wavelength is 780 nm and the output beam size is about 10 mm.

A half-wave plate (HWP) and a polarization beam splitter (PBS) adjust the polarization and power of the ultrafast amplifier, while a mechanical shutter (VS14S-2-ZM-0-R3, Uniblitz, USA) controls the exposure time to avoid photobleaching. The beam is spatially dispersed via a blazed grating with 600 lines/mm. The incident angle of the beam can be adjusted to ensure that the central frequency follows the optical axis. Temporal profile distortion induced by optical aberrations is of primary concern and must be compensated; therefore, a 32-element DM (MDM1-32S, Active Optical Systems, USA) with a resonance frequency greater than 500 Hz was positioned at the “spectral expansion” plane where individual spectral components are focused and separated from each other. At this spectral expansion plane, only the temporal profile is modulated and no additional spatial aberrations are generated. Due to the high peak power at the focus of the ultrafast amplifier, the DM would be damaged if it were simply placed at the spectral expansion plane. For this reason, two y-axis cylindrical lenses (i.e., curvature along the y-axis; L_{Cy1} and L_{Cy2}) relay the laser beam on the y-axis without focusing, while one x-axis cylindrical lens (L_{Cx1}) focuses each spectral component on the x-axis. By employing these lenses, the spectral separation on the x-axis remains, while the original beam size on the y-axis is maintained so that no damage to the DM occurs. Double passing a quarter wave plate (QWP) rotates the polarization by 90 degrees, allowing the beam to pass through a PBS with minimum power loss.

L₁ and an objective lens (W Plan-Achromat 40x/NA 1.0 or 20x/NA 0.8, Carl Zeiss, Germany) form a 4-f setup, and achieve temporal focusing at the front focal plane of the objective lens in an upright optical microscope (Axio imager 2, Carl Zeiss, Germany). By filtering the collected signal through a dichroic mirror and a short-pass filter, only nonlinear optical signals are imaged through an imaging lens (L_{imaging}) onto a high sensitivity electron multiplying charge-coupled device (EMCCD) camera (iXon EM + 885 EMCCD, Andor, UK) with TE cooling down to −90 °C. By controlling a motorized stage (H101A, ProScan™, Prior, UK) in the z-direction via a data acquisition (DAQ) card with a field-programmable
gate array (FPGA) module (PCI-7831R, National Instruments, USA), sequential sectioning images at different depths can be obtained automatically and assembled to reconstruct a 3D image.

Fig. 1. Optical setup of the temporal focusing-based multiphoton microscope with a wavefront sensorless AOS.

2.2. Wavefront sensorless image-based feedback control

Before activating the AOS, the overall system needs to be initialized and calibrated. In addition, the refractive elements in the microscope induce additional group velocity dispersion (GVD) that degrades the system performance. To compensate the GVD, the built-in grating compressor of the ultrafast amplifier can be adjusted to approach the optimal situation such that the temporal focusing plane coincides with the image plane of the imaging system; hence, high excitation efficiency can be achieved after calibration.

The feedback control mechanism for the AOS is based on the image intensity information via our modified hill-climbing algorithm. The overall image intensity or finite region of interest (ROI) can be treated as an image quality parameter; and it is assumed that this parameter would be maximized when the temporal profile distortion is minimized and no additional spatial aberrations are involved [33]. Other proposed image parameters can be found in [34] and used to evaluate image quality. The deflection of the DM is approximately proportional to the square of the applied voltage. To obtain both forward and backward deflections of the DM, a DC-bias voltage is used to maintain the DM at half of the maximum deflection as doing system calibration. Herein, the DM is arranged with six parallel segments which modulate the phase delay of the six corresponding spectral components at the spectral expansion plane. This configuration acts to compensate temporal profile distortion according to the image quality parameter. The correlation of the segments to the individual elements of the 32-element DM is shown in Fig. 2(a), while Fig. 2(b) illustrates the flow chart of the
modified hill-climbing algorithm. $M$ indicates the segment number. The maximum deflection of the DM is divided into $N$-steps. Each compensation cycle starts from the first segment and applies the voltage stepwise from zero to the maximum. The EMCCD camera captures the image at each voltage step, and then the overall image intensity is recorded. As the applied voltage reaches the maximum, the six segments of the DM are positioned according to the maximum image intensity. In our experiments, the final corrected image is taken after 2 compensation cycles in order to ensure control convergence. To accelerate the control loop, the frame rate of the EMCCD camera can be increased at lower exposure times and image ROI confinement to reduce the computation load.

![Diagram](image)

**Fig. 2.** (a) DM geometry and the six segments corresponding to individual elements. (b) Flow chart of the feedback control mechanism via the modified hill-climbing algorithm.

### 3. Experimental results and discussions

#### 3.1. Axial resolution compensation with wavefront sensorless AOS

A PMMA thin film doped with R6G dye (< 200 nm thick) was used as the specimen, for which the 20x/NA 0.8 objective lens was adopted. Also, an inhomogeneous layer of nail polish, applied to a cover glass and used as a temporal profile distortion source, was placed before lens $L_4$ in the optical path. At this position, the laser spot is focused into a line in a spectral expansion region. Herein, the wavefront sensorless AOS temporal focusing-based multiphoton microscope demonstrates its capability to refocus the axial excitation and maintain the axial resolution by scanning the thin film along the $z$-axis from top (negative) to bottom (positive). After averaging the TPEF intensities at different $z$-axis positions, an axial TPEF intensity profile can be obtained. Figure 3 shows the axial TPEF intensity profiles of the PMMA thin film without the added nail polish distortion (green), with the added distortion (red), and after the wavefront sensorless AOS compensation (blue). The circles represent the measured intensities at different $z$-axial positions, while the three solid lines are the fitted curves [6]. The distorted axial profile (i.e. red solid line) could not be fitted very well due to the irregular and asymmetric distortion. As can be seen by the distorted axial profile, the temporal distortion not only degrades the axial excitation profile but also asymmetrically decentralizes and shifts the excitation energy. The estimated axial resolutions of the three profiles are 3.6 μm, approximately 5.4 μm, and 3.8 μm, respectively, in full width at the half maximum (FWHM); hence, the axial confinement and TPEF intensity at the temporal focusing plane are recovered by the wavefront sensorless AOS. Furthermore, the distorted axial profile has an approximate 1.3 μm up-shift that refers to the GVD of the additional distortion [35]. The right side of the distorted axial profile is slightly flatter than the
left side, which might arise from the relatively high-order dispersion term effect. After implementing the AOS, the shift and asymmetry are both reinstated. Therefore, the experimental results demonstrate that the temporal focusing-based multiphoton microscope with the wavefront sensorless AOS not only refocuses the axial excitation symmetrically but also recovers the axial resolution with full TPEF intensity.

The temporal distortion compensation demonstrated here involved only relatively slight and low-order dispersion terms because of the limitation of the DM stroke range and element number. In order to extend the correction range, one could use a piezoelectric bimorph mirror [35] to compensate the fundamental GVD, and then employ a large element number DM or SLM to rectify higher-order temporal distortions. This combination is very similar to a conventional AOS that utilizes a tip-tilt mirror to eliminate $x$-$y$ tilt in Zernike terms, followed by an additional DM for compensating other-order aberrations.

![Axial TPEF fluorescence intensity profiles](image)

**Fig. 3.** Axial TPEF fluorescence intensity profiles of the thin film without distortion (green), with distortion (red), and after wavefront sensorless AOS compensation (blue), separately. Estimated axial resolutions for the three profiles are 3.6 μm, ~5.4 μm, and 3.8 μm, respectively, in FWHM.

### 3.2. Fluorescent thin film images with/without AOS

The PMMA thin film doped with R6G dye was used as a specimen to demonstrate the improvement via the wavefront sensorless image-based AOS. A $100 \times 100$ μm² (512 × 512 pixels) image was captured at a laser power of 12.3 mW illuminated on the specimen and an exposure time of 0.01 sec. We set $N$ equal to 10. It took less than 1.5 sec to complete 2 correction cycles. Figure 4(a) shows the TPEF image of the R6G thin film without the induced distortion. The area with many cracks is the ROI and was used for image contrast comparison. With the distortion induced by the cover glass with inhomogeneous nail polish, the temporal profile became distorted, reducing the excitation efficiency, as shown in Fig. 4(b). However, with the AOS compensation, not only is TPEF image intensity restored, but contrast in the cracks is also enhanced, as shown in Fig. 4(c). Figure 4(d) shows the TPEF intensity profiles at the red dashed line in Figs. 4(a)-4(c). The mean intensity is increased by 3.7-fold and the contrast of the cracks is obviously enhanced. The five locations, labeled 1-5, were used for contrast comparison by determining the relative intensity changes from the peaks to the valleys, and yielded enhancements of 2.8-fold, 3.7-fold, 2.2-fold, 5.3-fold, and 2.9-fold, respectively. The square of the final voltages applied on the six segments of the DM are shown in Fig. 4(e).
3.3. Imaging fluorescent beads at different depths

The specimen consists of a 1.0 μm fluorescent bead solution (3.6 × 10⁹ particle/ml) and agarose gel solution (1% v/v) with a volume ratio of 1. The specimen was confined in a small chamber created using 500 μm-thick adhesive tape as a spacer to separate the cover slip and microscope slide. Herein, the distortion sources included an agarose specimen with a high-concentration beads and a cover glass with an inhomogeneous layer of nail polish positioned before lens L₄, as previously described. These sources of distortion would worsen the temporal profile distortion at greater imaging depths. A 25 × 25 μm² (128 × 128 pixels) image was taken at a laser power of 17.5 mW illuminated on the specimen and an exposure time of 0.01 sec. The control parameters M and N were set as 6 and 10, respectively. Also, it took less than 1.5 sec to finish 2 correction cycles. Figure 5(a) shows the TPEF image at a depth of z = −19 μm (z = 0 μm set as the interface between the cover slip and the specimen) before AOS correction. The excitation efficiency decreased due to the distorted temporal profile; however, as Fig. 5(b) illustrates, image intensity is increased after AOS correction. Figure 5(c) shows the TPEF intensity profiles of the red dashed line of Figs. 5(a) and 5(b), and demonstrates intensity enhancements of 2.1-fold, 2.0-fold, and 2.1-fold for three main peaks from left to right, respectively. At the deeper depth of z = −60 μm in Fig. 5(d), individual beads situated closely together are hard to distinguish; however, as before, image intensity is enhanced with the AOS correction, as can be seen in Fig. 5(e). And finally, Fig. 5(f) features the TPEF intensity profiles of the red dashed line of Figs. 5(d) and 5(e), which show that the intensity enhancements of the two main peaks can be estimated as 1.7-fold and 1.8-fold for the left and right, respectively. Overall, intensity enhancement and better contrast can be achieved via the AOS compensation for fluorescent beads at the two different depths. However, the smaller intensity enhancement at greater imaging depths may be due to larger scattering and optical

Fig. 4. TPEF image of PMMA R6G-doped thin film: (a) without distortion, (b) with distortion, and (c) after wavefront sensorless image-based AOS compensation. (d) Intensity profiles of the red dashed line of Figs. 4(a)-4(c). Green line: without distortion; red line: with distortion; blue line: after AOS correction. Relative intensity changes of locations 1 to 5 are 2.8-fold, 3.7-fold, 2.2-fold, 5.3-fold, and 2.9-fold, respectively. (e) The square of the applied voltages at the six DM segments.
aberrations from the concentrated specimen that could not be fully compensated. Scattering due to turbid media, which is still a serious concern in widefield imaging, will restrict the overall improvement via wavefront sensorless image-based AOS, but it might be possible to partially mitigate this using novel image improvement techniques [11,36].

![Figure 5](image1.png)

(a) (b) (c)

Fig. 5. TPEF images of 1 μm fluorescent beads in agarose gel at the depth of −19 μm: (a) with distortion and (b) after AOS correction. (c) TPEF intensity profiles of the red dashed line in Figs. 5(a) and 5(b) and the intensity enhancements of 2.1-fold, 2.0-fold, and 2.1-fold for the three main peaks from left to right, respectively. TPEF images of fluorescent beads at the depth of −60 μm: (d) with distortion and (e) after AOS correction. (f) TPEF intensity profiles of the red dashed line in Figs. 5(d) and 5(e) and the intensity enhancements of 1.7-fold and 1.8-fold for the two main peaks for the left and right, respectively. Red line: with distortion; blue line: after AOS compensation.

4. Conclusions

In this study, a wavefront sensorless AOS was integrated into a temporal focusing-based multiphoton microscope. The feedback control signal of the AOS is derived from the maximization of the local image intensity via a modified hill-climbing algorithm. This then drives a DM in such a way as to eliminate the optical distortions which result in temporal profile distortions (the primary role of the implemented AOS), excitation efficiency reductions, and image quality degradation. However, due to the limitation of the DM stroke range and element number, only relatively slight and low-order dispersion terms were compensated here. Ultimately, there are three main outcomes of using the wavefront sensorless AOS compensation: 1) to restore axial resolution and excitation position; 2) to enhance the contrast and intensity of TPEF images of R6G-doped PMMA thin films; and, 3) to improve TPEF image quality of 1 μm fluorescent beads sealed in agarose gel at different depths. Therefore, these experimental results demonstrate that the temporal profile distortions in temporal focusing-based multiphoton microscopy can be compensated by the proposed wavefront sensorless AOS.
Acknowledgments

This work was supported by the National Science Council (NSC) in Taiwan with the grant numbers of NSC 101-2221-E-006-212-MY3 and NSC 101-2221-E-006-213-MY3.