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Ultrafast, large-field multiphoton microscopy based on an acousto-optic deflector and a spatial light modulator

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Abstract

We present an ultrafast, large-field multiphoton excitation fluorescence microscope with high lateral and axial resolutions based on a two-dimensional (2-D) acousto-optical deflector (AOD) scanner and spatial light modulator (SLM). When a phase-only SLM is used to shape the near-infrared light from a mode-locked titanium:sapphire laser into a multifocus array including the 0-order beam, a $136\ \mu\text{m} \times 136\ \mu\text{m}$ field of view is achieved with a $60\times$ objective using a 2-D AOD scanner without any mechanical scan element. The two-photon fluorescence image of a neuronal network that was obtained using this system demonstrates that our microscopy permits observation of dynamic biological events in a large field with high-temporal and -spatial resolution.

Multiphoton laser scanning microscopy is an ideal tool for exploring *in vivo* biological processes [1]. Its primary benefit in comparison with conventional fluorescence imaging techniques is that it has less photobleaching for areas of the sample away from the laser focus and has a lower scattering of the excitation beam due to the use of longer wavelength [2,3]. In conventional multi-photon laser scanning microscopy, galvanometric or resonant mirrors are used as scanners because they have large scan angles and no dispersion effect. However, galvanometric mirrors are relatively slow ($<1000\ \text{Hz}$), and resonant mirrors do not have the flexibility for scanning-rate selection. In addition, these mechanical scanners cannot rapidly access a specific point (random scanning) in the field of view (FOV) due to the mirrors' inertia.

Laser scanning achieved by two-dimensional acousto-optical deflectors (2-D AODs) overcomes these limitations, offering a broad range of selectable scanning speeds with high precision and stability [4,5]. However, acousto-optical deflectors (AODs) have two inherent features that have limited their application in multiphoton microscopy. One feature is dispersion—AODs generate a large temporal and spatial dispersion when ultrafast laser pulses propagate through the acousto-optical materials, especially because AODs typically use highly dispersive material such as a tellurium dioxide (TeO_2) crystal.

Dispersion causes pulse broadening and beam distortion, which reduce the signal-to-noise ratio and dramatically decrease the image resolution of multiphoton microscopy [6]. Several

effective dispersion-compensation methods have been reported by different research groups [6–8], among which Zeng's group reported that a single 45 deg-tilted prism can simultaneously compensate for both the temporal and spatial dispersion induced by a 2-D AOD [8]. Another disadvantageous feature of an AOD is its small deflecting angle. AODs normally have a deflecting angle of approximately 44 mrad. Thus, the FOV of AOD-based microscopy is about one-third of that of galvanometer-based microscopy. Using AODs, we obtain scanning flexibility and a fast scanning rate, but sacrifice FOV. However, large fields of view are often required in physiological studies, such as in studying the nonlinear interactions between synapses and in accurately locating the multitudes of stimuli [9]. The direct approach to solve the FOV problem is to increase the aperture of the AOD and the length of the acousto-optical crystal. But this approach further increases dispersion in addition to increasing fabrication cost. Other approaches have been used to compensate for the small deflecting angle and to increase the FOV. Bourdieu's group used the combination of a 2-D AOD and a 2-D resonant mirror set to achieve fast imaging in a large field [5]. Lechleiter *et al.* reported a video-rate two-photon system with a compound scanner comprised of a single AOD for fast scan and a galvo mirror for slow scan [10]. But their results showed that the FOV is expanded along only one direction. Moreover, using a compound scanning mechanism including a mechanical scanner decreases scanning flexibility. In this Letter, we report the development of ultrafast, large FOV, array-scanning multiphoton microscopy using a 2-D AOD scanner that completely overcomes the small-FOV shortcoming of conventional AOD scanning schemes.

For single-beam scanning microscopy with a 2-D AOD scanner and the tube lens that is designed for the objective, FOV is usually calculated by the formula

$$\text{FOV} = \frac{f_s \cdot \Delta\theta_{\max}}{M},$$

where $\theta_{\max}$ is the maximum deflecting angle of the AOD, f_s is the focal length of the scanning lens, and M is the magnification of the microscopic objective. Accordingly, it can be determined in terms of the AOD's deflecting angle that the FOV is approximately $66 \mu\text{m} \times 66 \mu\text{m}$ with a $40\times$ objective and $44 \mu\text{m} \times 44 \mu\text{m}$ with a $60\times$ objective when $f_s = 60 \text{ mm}$. Due to the small FOV, the microscope usually cannot be used to image dynamic biological events such as nerve-pulse propagation, which requires a large FOV in a network composed of several neurons. To address this issue, we employed a phase-only spatial light modulator (SLM) to shape the near-infrared (NIR) incident beam from a femtosecond (fs) laser into a specific multifocus array. Each focal point in this array scanned only a subregion of the FOV as in a single-beam scanning mode, and the entire FOV was scanned in parallel by the multifocus array. Array scanning was realized using the 2-D AODs to steer the laser beam to change the SLM's incident angle. Thus, the FOV of our microscope can be enlarged N -fold compared to that of the single-beam scanning technique in which N is the number of focal points. In addition to enlarging the FOV, a multifocus array setup also increases the image rate and the dwell time at each pixel to yield a high signal-to-noise ratio [11,12].

In many cases of biomedical imaging, the area of interest (e.g., a single cell with a long, thin protrusion) in a sample (e.g., a cell culture) occupies only several discrete regions of irregular shape and small areas in the large FOV, so scanning the entire FOV is excessive. With this AOD and SLM combined system, it is easy to produce a corresponding multifocus array to scan only the area of interest, thereby shortening the scanning time and reducing the risk of photodamage.

Figure 1 is a schematic of the ultrafast, large-field multiphoton microscope composed of a phase-only SLM, a 2-D AOD scanner, and our custom-built optical microscope that consists of several Olympus microscopic components (e.g., 60× Olympus water-immersion objective, NA 1.0) and optical stages from ThorLabs. A Ti:sapphire laser (Spectra-Physics, Tsunami pumped by a 10 W Millennia) was tuned at a wavelength of 830 nm and a pulse duration of 100 fs. A 2-D AOD scanner (AA Opto-electronic, DTSXY-400-810, A-A) was used for the ultra-fast scan. The center frequency of the 2-D AODs was 100 MHz, and the range of the scan frequency was 80–120 MHz. An uncoated, 45 deg tilted SF11 prism with an apex angle of 60 deg (Newport) was used to simultaneously compensate for the temporal and spatial dispersion of the 2-D AODs [8]. After being scanned by the 2-D AOD scanner, the laser beam was expanded and collimated to slightly overfill the phase-only SLM (Holoeye Pluto, NIR, 1920 × 1080 pixels, 8 bits), which is capable of completing 2π -phase modulation at each pixel with a 60 Hz refresh rate. The SLM can be used to dynamically produce specific patterns with computer-generated phase-only holograms. An accurate phase pattern can be generated by employing phase-retrieval algorithms such as the classic Gerchberg–Saxton algorithm, or by directly utilizing the Holoeye application software. A Fourier lens with a focal length of 200 mm transfers the light that is phase modulated by the SLM into a multi-focus array at the back focal plane of the tube lens (the largest focal array is 15 mm; when the array is scanned, the effective imaging field is approximate 22.5 mm, approximately the size of the microscope's FOV at that plane). The front focal plane of the tube lens is located at the entrance of the microscope objective. The objective then produces one or a series of multifocus arrays at the sample plane. The multiphoton fluorescence signal is separated from the NIR excitation light by a dichroic mirror (Semrock, FF665-Dio2) and is recorded by an EMCCD camera (Andor, DU-888E-C00-#DZ, 1024 × 1024, 9 fps).

To make full use of the laser power, instead of blocking the 0-order beam [13,14], we utilized the 0-order beam to form the central focal point in the multifocus array. To use the 0-order diffraction beam, the intensity of the 0-order diffraction light must have approximately the same power as that of each diffraction beam. So the number of foci of the multifocus array is related to the diffraction efficiency of the SLM, which can be determined by specifically designed phase-only holograms for the SLM [15].

The diffraction efficiencies are explicitly dependent on the polarization state of the normally incident light [16]:

$$\eta_{+1} = \frac{1}{2} [1 - S] \sin^2 \left(\frac{\pi \Delta n d}{\lambda} \right),$$

where, η_{+1} is the diffraction efficiency of the 1-order, λ is the vacuum wavelength of incident light, Δn is the linear birefringence, d is the grating thickness, and S is the normalized Stokes parameter corresponding to the incident light's ellipticity. In our case of using a 3×3 multifocus array, using the Holoeye application software to generate the phase-only holograms, the diffraction efficiency could be tuned by changing the polarization of the incident light so that the 0-order beam would have approximately the same power as the other eight beams of the multifocus array that were formed by the SLM diffracted beams. A 3×3 multifocus array was designed and applied in our experiment (Fig. 2). By slightly tuning the polarization of the light, a multifocus array with approximately 6.5 mW per focal point was achieved. It enlarged the FOV nine-fold in comparison with a single beam-scan technology with the 2-D AOD scanner. The array of nine foci was raster scanned across the FOV by the 2-D AOD scanner driven by a NI-PCI6536 DAQ card. The $1/e^2$ widths of the radial and axial point spread function were estimated by scanning a 0.2 μm fluorescent bead to be 0.69 μm and 2.2 μm , respectively.

The effectiveness of the developed ultrafast, large-field multiphoton microscope was demonstrated by imaging the fresh pollen grains from the flower of the Yellow Leaf Oleander (Fig. 3) and a cultured neuronal network stained by DiO. Figure 4 shows the two-photon fluorescence image of the neuronal network. The size of the FOV was approximately $136\ \mu\text{m} \times 136\ \mu\text{m}$ with a scanning rate of 400 f/s. It clearly displayed the structure with high lateral resolution and signal-to-noise ratio.

In conclusion, we demonstrated that parallel excitation using a multifocus array generated by an SLM in multiphoton microscopy with a 2-D AOD scanner resolves the conflict between image rate and FOV. Furthermore, through scanning the multifocal spots produced by the SLM in parallel, the number of usable resolvable spots of the AODs can be increased n times, where n is the number of focal spots. By adjusting the polarization of the incident beam to the SLM, the 0-order beam can have a power similar to that of each high-order beam and thus be used in the multifocus array. It should be noted that the diffraction pattern change (e.g., from 3×3 to 5×5) will require a retune of the input polarization and a new array-generation algorithm so that the 0-order beam will have power equal to the diffracted beams. In addition, it is feasible to simultaneously image multiple areas of interest in a large FOV by uploading the required phase mask of the multifocus array generated according to the shape of the areas of interest and by designing a specific scanning path since the two scanning mechanisms are identical. The unique feature of our design has great potential for use in observation of dynamic signal transportations, such as the nerve pulse propagation in a neuronal network.

Acknowledgments

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Fig. 1.

(Color online) Schematic of the fast, large FOV multi-photon microscope with an SLM and a 2-D AOD scanner. The prism is for compensating the dispersion introduced by AODs. An uncoated, 45 deg-tilted SF11 prism with an apex angle of 60 deg is used to compensate for the temporal and spatial dispersion of the 2-D AODs. The total path length between the prism and the 2-D AODs is 18.5 cm. The pulse width is 125 fs after AODs. M1 and M2 are beam-steering mirrors. The focal lengths of L1 and L2 are 30 and 100 mm, respectively. The SLM on the focal plane of the Fourier lens modulates the incidence beam to generate a multifocus array including the 0-order beam on the focal plane of the tube lens. The focal length of the tube lens is 180 mm. The distance from the objective back focal plane (BFP) to the SLM is 760 mm. The polarization of light can be tuned by the $\lambda/2$ plate. (Laser, an fs laser; M1, M2, and M3: gold mirror; Camera, EMCCD).

Fig. 2. (Color online) Design of a 3×3 multifocus array, (a) multifocus array of the high-order diffraction beams, (b) focal point of the 0-order diffraction beam, and (c) compound multifocus array.

Fig. 3. (Color online) Two-photon fluorescence intensity image of fresh pollen grains from the flower of the Yellow Leaf Oleander, obtained by recording the autofluorescence with an exposure time of 3 ms. Scale bar is 20 μm .

Fig. 4. (Color online) Two-photon fluorescence intensity image of a cultured neuronal network (3-day cultured chick fore-brain neurons harvested at embryonic day 7) live-cell stained with DiO. Cell A, Cell B, and Cell C form a linear neuronal circuit connected by axons (yellow arrows). Scale bar is 20 μm .