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Addressable, large-field second harmonic generation microscopy based on 2D acousto-optical deflector and spatial light modulator

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Abstract

We present an addressable, large-field second harmonic generation microscope by combining a 2D acousto-optical deflector with a spatial light modulator. The SLM shapes an incoming mode-locked, near-infrared Ti:Sapphire laser beam into a multifocus array, which can be rapidly scanned by changing the incident angle of the laser beam using a 2D acousto-optical deflector. Compared to the single-beam-scan technique, the multifocus array scan can increase the scanning rate and the field-of-view size with the multi-region imaging ability.

1 Introduction

The second harmonic generation (SHG) microscope, with its unique imaging contrast, has become a powerful tool for observing biological processes in vivo [1]. SHG is a nonlinear

optical phenomenon similar to two-photon excited fluorescence (TPEF). Both techniques provide the same intrinsic 3D resolution, but SHG is a coherent process, in which two lower-energy photons are up-converted to exactly twice the incident frequency (or half the wavelength) rather than a process that involves the excitation of molecules such as generation of fluorescence (including multiphoton). Thus, SHG can substantially reduce photobleaching and phototoxicity [2]. The recent reports in the field of SHG application have mainly focused on visualizing polar molecules organized in noncentrosymmetric structures such as collagen fibers, microtubules, and contractile proteins of muscle fibrils [2]. However, conventional SHG microscopy cannot meet the scanning speed necessary to observe dynamic biological processes, such as the contraction of sarcomeres within a cardiomyocyte, which requires an imaging time of a few ms. Several fast imaging methods have been reported by different research groups [3, 4]. Minoru Kobayashi's group reported an SHG microscope based on spinning-disk-scanning technique with a frame acquisition time of up to 33 ms [3]. However, the spinning-disk scanner had a concomitant loss of incident power of nearly 75 % for generating the pattern of excitation foci [5]. Parker's [4] group introduced a resonant scanning mirror that permitted a frame rate of 30 Hz. The resonant mirrors did not have the flexibility for scanning-rate selection. In addition, these mechanical scanners could not rapidly access a specific point of interest (random scanning) in the field of view (FOV) due to the mirror's inertia. Laser scanning achieved by two sequential, orthogonally oriented acousto-optical deflectors (2D AODs) with ultrafast scanning speed overcomes these limitations, offering a broad range of selectable scanning speeds with high precision and stability [6, 7]. Peter Saggau's group reported a fluorescence microscope that achieved a random access rate of up to 200K samples per second and a frame rate up to 5 kHz using a two-AOD scanner. However, two inherent features of AODs have limited their application in nonlinear optical microscopy: dispersion and small deflection angle. In this letter, we report the development of an addressable, high-flexibility, large FOV SHG microscope using a 2D-AOD scanner and a spatial light modulator that completely overcomes the small-FOV shortcoming of conventional AOD-scanning schemes.

2 Method and experiment

There are typically two kinds of methods for resolving the small-deflecting-angle problem: one is the combination of a 2D AOD and a 2D-resonant-mirror, the other is the application of a compound scanner comprising a single AOD for fast scan and a galvo mirror for slow scan [8]. However, using resonant mirrors causes less scanning flexibility, and results using the compound scanner show that the FOV is expanded along only one direction. In this study, we proposed using a multifocus-scanning technique with our SHG microscope to enlarge the FOV and to increase the scan rate. A phase-only SLM was used to shape the near-infrared (NIR) incident beam from a mode-locked Titanium:Sapphire laser into a designed multifocus array based on the sample's region(s) of interest. The change of the array configuration, including the number of foci and the shape of the array, can be realized by uploading the corresponding phase mask to the SLM without the change of any hardware. Each focal point in this array scans only a subregion of the FOV as in a single-beam-scanning mode, and the entire FOV is scanned in parallel by the multifocus array. Theoretically, the scan of the multifocus array can be achieved by dynamically uploading a series of phase patterns corresponding to the focal points at various locations to be scanned. But the response time of commercially available SLMs, including the one used here, is too slow (less than 100 Hz) to provide a sufficiently high frame rate for imaging dynamic biological processes. For example, to obtain an image of 256×256 pixels using an array of 3×3 focal points generated by an SLM with a 60 Hz refresh rate, we must upload as many as 7,281 phase patterns to the SLM, which takes about 121 s. Therefore, a scan of the multifocus array cannot be practically achieved using the SLM [9]; it must be realized using another mechanism. It was accomplished in our system by scanning the SLM's incident

beam. When the incidence angle to the SLM is very small, the translation of the focus array is proportional to the change of the incidence angle [10]. In our system, the array-scanning was realized using a 2D AOD to steer the laser beam to change the SLM's incident angle. Thus, the FOV and image rate of our microscope can be, respectively, enlarged and increased N -fold compared to that of the single-beam-scanning technique, in which N is the number of focal points.

To resolve the problem of dispersion of AOD, several methods have been reported [11–13]. Especially, Zeng's group reported that a single 45° tilted prism (SF10) simultaneously compensated for both the temporal and the spatial dispersion induced by a 2D AOD [13]. In their report, the selected distance between the prism and the 2D AOD was 35 cm. Here, to decrease the distance between the prism and the 2D AOD, we use an SF11 prism with a higher dispersion coefficient to simultaneously compensate for both the temporal and the spatial dispersion induced by the 2D AOD.

Figure 1 is a schematic of the addressable, ultrafast, large-field SHG microscope composed of a phase-only SLM, a 2D AOD scanner, and our custom-built microscope that consists of several Olympus-microscopic components (e.g., $60\times$ Olympus water-immersion objective, NA 1.0) and optical stages from ThorLabs. Forward second harmonic signal is collected by an objective lens ($63\times$ Zeiss oil-immersion objective, NA 1.4). A Ti:Sapphire laser (Spectra-Physics, Tsunami pumped by a 10 W Millennia) with a pulse duration of 100 fs is tuned at a wavelength of 810 nm. A 2D AOD scanner (AA Opto-electronic, DTSXY-400-810, A–A) is used for an ultrafast scan. The center frequency of the 2D AOD is 100 MHz, and the range of the scan frequency is 80–120 MHz. An uncoated, 45° tilted SF11 prism with an apex angle of 60° (Newport) is used to simultaneously compensate for both the spatial and the temporal dispersion of the 2D AOD. In our experiments, the incident angle and distance between the prism and the AOD are optimized to 57° and 18.5 cm, respectively. We determined the distance between the prism and the AODs by continuously measuring the pulse width after AODs using a pulse autocorrelator with an error of ± 5 fs from Spectra-Physics as the distance was scanned. When the pulse width had a minimum value (in our case it was 125 fs) the corresponding distance was taken for the setup (in our case it was approximately 18.5 cm). After being scanned by the 2D AOD scanner, the laser beam is expanded and collimated to slightly overfill the phase-only SLM (Holoeye Pluto, NIR, $1,920 \times 1,080$ pixels, 8 bits), which is capable of completing a 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 (GS) 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 multifocus array at the back focal plane of the tube lens (the largest focal array is 15 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 array of foci is raster-scanned across the FOV by the 2D AOD scanner driven by a NI-PCI6536 DAQ card. The second harmonic signal is separated from the near-infrared excitation light and fluorescence by a notch filter mirror (Semrock, FF01-405/10-25) and is recorded by an EMCCD (Andor, DU-888E-C00-#DZ).

We have designed a unique multifocus pattern (Fig. 2a), the corresponding phase pattern of which is calculated using Holoeye application software. The uniqueness is that the 0-order light is located at the center spot of the multifocus pattern so that the 0-order light and the high-order light generate a compound 3×3 multifocus array (Fig. 2b). An FOV of $136 \mu\text{m} \times 136 \mu\text{m}$ is generated, which is ninefold greater than that in a single-beam-scan technology

with the 2D AOD scanner. Moreover, compared to other microscopic techniques based on the SLM [9, 14, 15], there is no dark area that is generated by blocking the 0-order light, and the efficiency of laser use is improved because the 0-order light is used effectively in our design.

The effectiveness of the proposed addressable, large-field SHG microscopy was tested by scanning a “3”-shape microstructure filled with ZnO solution and recording the SHG image of the ZnO solution with an EMCCD camera. Fast raster scanning of the multifocus array was carried out by the 2D AOD scanner, and the forward SHG signal was captured in parallel by the EMCCD camera, as shown in Fig. 3a. In our experiments, the laser power reflected by the SLM before the pure phase pattern is uploaded to the SLM is 65 mW. By tuning the polarization orientation of the incident light to change the ratio of the high-order diffraction light intensity to that of the 0-order diffraction light after uploading the pure phase pattern to SLM, a multifocus array with approximately 6.5 mW per focal point was achieved.

To further confirm the flexibility of our microscopy, we imaged a biochip manufactured with polydimethylsiloxane (PDMS) that featured microslots (10 μm wide, 40 μm deep, and 63 μm long) filled with ZnO nanoparticle solution. The distance between the adjacent slots was approximately 54 μm . We changed the row distance of the multifocus array [10] and the scanning parameters of the 2D AOD scanner (maintaining the x -scan while decreasing the y -scan range) based on the three regions of interest, where the slots were located, to ensure that only the interest regions would be scanned. While the three rectangular regions were scanned in parallel by the 2D AOD scanner, SHG signal was simultaneously recorded by the EMCCD camera. The SHG image of the three microslots is shown in Fig. 4.

3 Discussion and conclusion

We demonstrated a flexible, large-field SHG microscopic technique based on a 2D AOD scanner and an SLM for high-speed, addressably scanning and simultaneously imaging multiple regions of interest. The compound multifocus array design was shown to work with a 3×3 multifocus array. The FOV was enlarged from 45 μm $9 \times 45 \mu\text{m}$ to 136 μm $9 \times 136 \mu\text{m}$, and the maximum scan rate was improved from 125,000 to 1,125,000 points/s. The 3×3 array with equal interfocal distance shown in Fig. 2b is a typical array; an array with unequal interfocal distances, such as the one used to obtain the image in Fig. 4, can be generated as easily as the typical one (Fig. 2). This kind of array, which can be designed based on regions of interest to achieve the flexibility of our SHG microscope, can be realized simply by uploading the corresponding pure phase pattern without a change of hardware. This phase pattern can be easily produced numerically in terms of the position, shape and size of the area of interest. In addition, through scanning in parallel the multifocal spots produced by SLM, the number of the usable resolvable spots of AODs can be improved N -fold compared to that of the single-beam-scanning technique, in which N is the number of focal spots. In fact, our current system design can be used only with a 3×3 focal array. In the future, we will explore the design of specific phase patterns for the SLM so that a denser array, such as 5×5 , can be used. Therefore, our SHG microscope is an ideal tool for observing high-speed biological processes.

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Fig. 1.

Schematic of the addressable, large-field SHG microscope based on a 2D AOD scanner and SLM. The 2D AOD scanner comprises an x -scanner and a y -scanner. The effective distance between the prism and 2D AOD scanner is 18.5 cm. The SLM is on the focal plane of the Fourier lens and modulates the incident beam to generate a multifocus array on the focal plane of the tube lens. The polarization of light can be tuned by the $\lambda/2$ plate. *Laser* Model-locked laser, $L1$ and $L2$ expanding beam Lens; $M1$, $M2$, $M3$ and $M4$ gold film mirrors, *Camera* EMCCD

Fig. 2. Design of a 3×3 multifocus array. **a** The designed multifocus pattern formed by the high-order diffraction beams, **b** the compound multifocus array, in which the red spot is the 0-order diffraction light

Fig. 3. The SHG image of a microslot structure of the number “3” filled with ZnO nanoparticle solution. *Scale bar* 20 μm

Fig. 4.
The SHG image of a biochip featuring microslots filled with ZnO nanoparticle solution with only the corresponding three scanned rectangular subregions enclosed by *red rectangles*.
Scale bar 20 μm