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Addressable discrete-line-scanning multiphoton microscopy based on a spatial light modulator

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Abstract

We developed a novel addressable discrete-line-scanning multiphoton microscope with high lateral and axial resolutions based on a spatial light modulator. Our discrete-line-focus design eliminates the cross talk that occurs in conventional one-dimensional line-scanning multiphoton microscopies. Additionally, a phase-only spatial light modulator is able to scan only a sample's target area by generating a specific discrete line focus according to the shape and location of the target area. Compared with other multiphoton microscopies, this technique shortens scanning time and minimizes photodamage by concentrating scanning energy and dwell time on the area of interest.

Multiphoton laser scanning microscopy offers the possibility of three dimensionally imaging thick specimens with high resolution without confocal pinholes [1]. But because of the low scanning rate, it cannot be applied to imaging the dynamic behavior of samples. One of the first methods for improving scanning rate was line scanning, in which the excitation laser is focused with a cylindrical lens to create a line focus on the sample plane, making scanning in one lateral direction unnecessary [2]. Line-focus can, however, result in cross talk due to the lack of confocal gates [3,4]. Interference on out-of-focus planes occurs between adjacent points when they are too close, degrading the resolution of the imaging system. In recent years, multifocal multiphoton microscopy (MMM) has emerged as a useful tool for high-speed image acquisition with high resolution [4–7]. In MMM, a single femtosecond (fs) laser beam is spatially or temporally split into N subbeams to generate multiple foci on the sample plane. The scan area is equally divided into N subareas, and each subbeam simultaneously scans one of the subareas. Scanning in a parallel manner combined with a two-dimensional (2D) camera that records the entire field in parallel, the acquisition time can be greatly shortened, typically by a factor of N . This permits studying the dynamic behavior of three-dimensional biological samples within an acceptable duration with high resolution (due to the confocal gating effect) and low risk of photodamage. Scanless MMM has been proposed [8], in which the number of focal points is limited by the laser power, and thus the imaging region is restricted. It is suitable for research such as that described in [8], in which small number of imaging points is sufficient. When large number of imaging points (e.g., large imaging area) is required, scanning becomes necessary.

Compared with the line-scanning technique, 2D scanning MMM still has one drawback: 2D scanning is always slower than one-dimensional (1D) scanning. In this study, we developed a novel addressable discrete-line-scanning (ADLS)-MMM, which, while operating under a 1D scanning mode, completely overcomes the shortcomings of conventional line-scanning schemes in resolution. Moreover, we expect the addressable capability to be a solution to the insufficient energy characteristic of all MMM setups when a large number of parallel foci are required. In conventional MMMs, to ensure sufficient power for each subbeam, the number of foci must be decreased, which increases the scanning range, and thus the scanning time for the same field of view (FOV). However, in many cases, the area of interest (e.g., a single cell) in a sample (e.g., a cell culture) occupies only a small portion of the FOV, and it is not necessary to scan the entire FOV. Thus, if scanning only the area of interest, we do not need to generate a large number of foci, and the scanning time can be shortened. In our MMM design, using a phase-only spatial light modulator (SLM), the size and location of the scanning area can be precisely selected to match the area of interest within a large FOV, achieving fast imaging with high lateral and axial resolution.

Figure 1 is a schematic of our ADLS-MMM. The beam from a mode-locked Ti:sapphire laser (Spectra-Physics Tsunami) is deflected by a two-axis galvo scanner (Cambridge Tech) driven by a NI-PCI6115 DAQ board. A pair of achromatic lenses (L1 and L2) with focal lengths of 30 and 100 mm, respectively, is used to expand the beam to slightly overfill the surface [i.e., liquid crystal on silicon (LCoS)] of the SLM (Holoeye PLUTO, 1920×1080 pixels, 8 bit phase quantization, 60 Hz refresh rate). The SLM is able to produce an arbitrary intensity pattern with a phase-only computer-generated hologram (CGH), which can be calculated using phase retrieval algorithms such as the classic Gerchberg-Saxton (GS) algorithm [9] or directly using Holoeye application software. Before reaching the SLM, the incident light is tuned by a polarization beam splitter (PBS) and a half-wave plate to obtain the optimal polarization that the SLM requires to achieve the highest modulation efficiency and accuracy. A Fourier lens (L3) with a focal length of 200 mm transfers the phase-modulated light from the SLM into multiple foci at the front focal plane of the tube lens in our custom-built microscope. A zero-order blocker is placed on the focal plane to block the unwanted zero-order bright spot. The blocker is made of a small piece of black aluminum foil that is stuck to a glass coverslip. A water-immersion objective (Olympus LUMplanFL, 60 $\times$, NA = 1.0) then focuses the multiple beam onto the sample plane. The multiphoton fluorescence signal is separated from the near-IR excitation light by a dichroic mirror (Semrock, FF665-Dio2) and recorded by an EMCCD (Andor DU-888E-C00-#DZ).

In our experiment, the process of generating multiple foci mainly includes the following three steps: (1) Design the required focal pattern numerically with Matlab, and create a matrix to represent the focal plane and pixels corresponding to the focal points are assigned a value of one and the remaining pixels are assigned a value of 0. In our case, the size of the matrix was selected as 310×310 pixels, corresponding to a sample plane size of $230 \mu\text{m} \times 230 \mu\text{m}$ that approximately matches the FOV of the imaging system. (2) Calculate the pure-phase CGH in terms of the matrix by using Holoeye application software, and upload the calculated CGH onto the LCoS screen of the SLM. (3) Illuminate the LCoS screen with a collimated fs laser beam at approximate 4° , and transform the CGH into the desired foci pattern with a Fourier lens. The design of the discrete focusing line is depicted in Fig. 2. The dotted grids represent the matrix mentioned in the description of the multiple-foci-generating process (step 1). The distance between two adjacent elements in the matrix matches the resolution of the imaging system. We consider a line as a series of resolvable points (the red spots) with the distance between two adjacent points equal to the resolution, as shown in Fig. 2(a). To avoid the cross talk, we did not create a line of foci; instead, we generated a saw-tooth-shaped pattern of foci by periodically shifting a group of foci that would be linearly arranged if a line focus were designed. For example, the second focal

point was shifted relative to the first one by a unit distance that was compatible with the minimum distance without cross talk (in our case, it was about three grids) and the third focus, by two units. Repeatedly, the fifth and sixth spots were shifted in the same way relative to the nonshifted fourth spot. In this way, all the resolvable spots composing the line were completely separated, as shown in Fig. 2(a), right. Suppose that the orientation of the original line is along the y axis, and the shifting direction is along the x axis. The distance between any two adjacent spots can be easily changed. For example, the shifting strategy in Fig. 2(b) enlarges the intervals in both the x and the y axes compared with the spots pattern in Fig. 2(a). Figure 2(c) is an example of designed discrete line. The scanning direction is perpendicular to the orientation of the discrete line. During a 1D scan, each focus scans one line in the sample. The distance between the lines of two adjacent foci in the sample is equal to the lateral resolution of the microscope, resulting in a scan of the sample with the highest resolution but without the loss of sectioning capability because the confocal gate is retained.

The length of the discrete line and its orientation in the entire FOV of the CCD camera are easy to adjust since the SLM can be reprogrammed to upload different CGHs by computer. The linear scan perpendicular to the line orientation can be achieved using the two-axis scanner. This permits the MMM to be addressable. For example, if a sample of interest of 50 μm in length is located at a coordinate of (a, b) in the FOV, we can accordingly set the discrete line with the same length and linearly scan it at only this location less than 1 ms. The total acquisition time of a multiphoton image is decided only by the sensitivity and readout time of the CCD.

The effectiveness of the proposed ADLS-MMM system was tested by scanning an adult rat cardiomyocyte and recording the two-photon image of the stained α -actinin with a EMCCD camera. We set the power of the pump laser at 7.5 W. The pulse width was 100 fs, the repetition frequency was 80 MHz, and the wavelength was 765 nm. First, the location of the target cell in the FOV was determined with white-light illumination. Once the target cell and its orientation were determined, we input key parameters to our custom-designed Matlab program to generate the corresponding CGH based on Holoeye application software. The discrete line was composed of 100 foci, and the power of each was 0.25 mW. Fast 1D scanning was carried out by the two-axis galvo scanner, and the two-photon signal was captured in parallel by the EMCCD camera, as shown in Fig. 3(a). The scanning rate was 2000 fps, more than 1000-fold faster than a conventional single-beam raster scan. However, the final EMCCD camera (100 ns per pixel). The size of the entire FOV is approximately $230 \mu\text{m} \times 230 \mu\text{m}$. Figure 3(a) shows the two-photon image acquired in an exposure time of 1 s without electron-magnified gain loaded on the EMCCD camera. It displays the sarcomere structure with a high lateral resolution and signal-to-noise ratio. By mechanically moving the sample along the axial direction with a step length of 2 μm , we recorded seven two-photon images of the cell in sequence. Figure 3(b) shows the cut images extracted from the FOV. The z scanning results demonstrate the sectioning capability of our ADLS-MMM scheme. We also measured the lateral and axial resolutions, to be 0.5 and 1.3 μm , respectively, after deconvolution.

In conclusion, we developed a novel addressable discrete-line-scanning multifocal multiphoton microscope with high lateral and axial resolutions based on a spatial light modulator. This microscope works in a fast 1D scanning mode, which completely overcomes the cross talk that characterizes conventional line-scanning MMMs. Because a discrete line is generated by a programmable SLM, the interfocal distance between adjacent foci can be set arbitrarily. The scanning strategy is akin to that of the spinning-disk-based MMM in which, upon rotation of the disk, each microlens scans one line in the sample, and the distance between adjacent lines remains well below the lateral resolution, assuring homogeneous scanning of the sample [5,10]. Whereas in our ADLS-MMM, the use of SLM

activates the addressability of MMM. By precisely designing the multiple foci according to the size and position of the area of interest, we can concentrate all the scanning energy and dwell time on that area of the sample. In this way, scanning time can be shortened compared with other MMMs, and the photodamage can be kept to a minimum. Moreover, the addressability and reprogrammability of our ADLS-MMM allows for additional applications compared with other MMMs: FOV may be switched quickly and discretionarily. For instance, in biology one may expect to observe changes that occur only occasionally in cultured live cells. By employing the ADLS-MMM, one can simultaneously observe a number of cells within a large FOV using single-beam scanning or 2D multiple-beam scanning (both can be achieved without changing the setup because the SLM can produce any foci pattern). Once the desired change occurs in a particular area, one can quickly switch on a very small FOV within that area, and then rapidly scan in 1D and record the dynamic process of the biological change. This cannot be achieved by other MMMs. In addition, the ADLS-MMM can be applied to simultaneously image two or more samples by using multiple discrete lines.

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Fig. 1.
(Color online) Schematic optical setup of the ADLS-MMM. PBS, polarized beam splitter; GS, 2D galvo scanner; SLM, spatial light modulator; zero-order beam blocker, a small piece of black-painted aluminum foil on a glass slide used to block the unwanted zero-order bright spot; L1, L2, beam expander ($f=30$ and 100 mm, respectively); L3, scanning lens ($f=200$ mm); L4, tube lens ($f=180$ mm); Objective, water-immersion objective, $60\times$, $NA=1.0$; M1, M2, M3, M4, mirrors.

Fig. 2.
(Color online) Design of the discrete lines with (a) shorter distance, (b) longer distance between adjacent points, and (c) image of the line.

Fig. 3.
(a) Two-photon image of adult rat cardiomyocyte in full FOV obtained by ADLS-MMM,
(b) axial-stack two-photon images of the cell.