Simultaneous multiplane confocal microscopy using acoustic tunable lenses

Martí Duocastella,* Giuseppe Vicidomini, and Alberto Diaspro

Nanophysics, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy
*marti.duocastella@iit.it

Abstract: Maximizing the amount of spatiotemporal information retrieved in confocal laser scanning microscopy is crucial to understand fundamental three-dimensional (3D) dynamic processes in life sciences. However, current 3D confocal microscopy is based on an inherently slow stepwise process that consists of acquiring multiple 2D sections at different focal planes by mechanical or optical z-focus translation. Here, we show that by using an acoustically-driven optofluidic lens integrated in a commercial confocal system we can capture an entire 3D image in a single step. Our method is based on continuous axial scanning at speeds as high as 140 kHz combined with fast readout. In this way, one or more focus sweeps are produced on a pixel by pixel basis and the detected photons can be assigned to their corresponding focal plane enabling simultaneous multiplane imaging. We exemplify this method by imaging calibration and biological fluorescence samples. These results open the door to exploring new fundamental processes in science with an unprecedented time resolution.

OCIS codes: (180.0180) Microscopy; (170.1790) Confocal microscopy; (110.1080) Active or adaptive optics.

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1. Introduction

Confocal microscopy has become the standard imaging technique in fields as relevant as materials science and life sciences [1,2]. The key aspect of confocal techniques is the ability to reject out of focus light, enabling one to obtain optical sections of a sample. Contrary to wide-field microscopy, confocal methods use point by point scanning for image formation. This can limit the speed performance of confocal microscopy which is a critical factor when studying the temporal dynamics of fast processes in areas such as neuroscience or chemistry. To maximize the amount of spatiotemporal information retrieved, two different strategies are commonly used. The first one consists in simultaneously using multiple point sources. Examples of this strategy include spinning disk confocal microscopy [3] or, more recently, digital micro-mirror devices [4]. Alternatively, one can simply scan the beam across the sample at increasing speeds. An example of this approach is the use of resonant galvanometric mirrors [5]. Both strategies have been proven as effective to decrease the acquisition time of 2D optical sections. However, in order to capture a 3D image multiple 2D sections must be acquired each at a different focal plane. This stepwise approach requires mechanical z-axis translation of the focus in between sections inherently limiting the acquisition speed in 3D confocal microscopy. For instance, widely used piezoelectric z-scanners are fundamentally limited by the inertial response of the objective to speeds below 100 Hz [6]. In addition, the physical movement of mechanical scanners can induce vibrations on the sample that affect image quality. Higher z-scanning speeds can be achieved without moving sample or objective via remote focusing [7], but this approach is difficult to implement and it is still limited by inertia.

An interesting alternative to conventional z-scanners are electronic tunable lenses [8]. These lenses allow to move the focal point of the objective lens along its optical axis, thus the axial scanning can be achieved without mechanically moving the specimen or the objective. Since lens tunability is usually based on changing the curvature of small and light systems such as polymeric membranes [9] or liquid droplets [10], inertial effects can be minimized allowing axial scanning speeds as high as 500 Hz [11,12]. Successful applications of tunable lenses include confocal microscopy [13,14] and other imaging modalities such as endoscopy [15], light sheet microscopy [16] and two-photon microscopy [17]. However, the need to physically change the lens curvature still imposes a fundamental limitation in terms of the speed such z-scanning systems can reach.

Here, we circumvent these speed limitations by integrating an acousto-optic lens in a commercial confocal system to achieve axial scanning at 140 kHz without the need of mechanically moving parts. Such high speed enables one to capture extended depth of field (EDOF) images as well as to acquire multiple and selectable focal planes simultaneously,

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thereby providing a different and much faster approach to 3D confocal microscopy than previously scanning systems.

2. High-speed z-scanning confocal microscopy

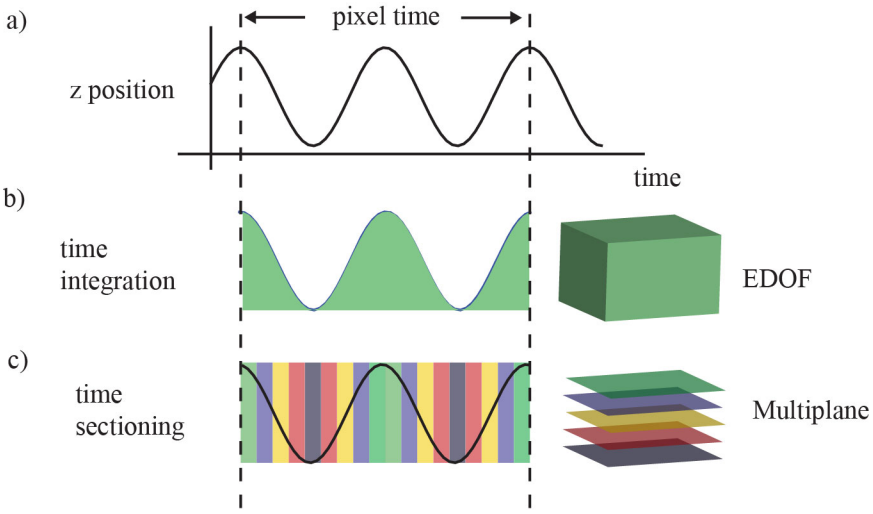

Fig. 1. Principle of operation of the high-speed z-scanning confocal microscope. a) Due to the high axial scanning speed enabled by the optofluidic lens, several axial scans are recorded within a pixel exposure. b) The sum of all photons providing from different focal planes forms an image with an extended depth of field (EDOF). c) By using a fast acquisition card and appropriate synchronization, photons arriving at different times can be sorted according to their corresponding focal plane. Such spatiotemporal multiplexing results in a user-selectable number of focal planes.

The high-speed z-scanning capabilities of our confocal system are enabled by a tunable acoustic gradient (TAG) lens [18]. This device consists of a fluid-filled cylindrical resonant cavity that uses sound energy to create a periodic modulation in the fluid refractive index. As such, when the TAG lens is driven with a signal of 143 kHz the lens focal length periodically varies between positive (converging) and negative (diverging) values [Fig. 1(a)]. By placing the lens in the back focal plane of the objective lens (or in a conjugate plane), the system focus can be axially scanned at the lens driven frequency [19]. Since no mechanical moving parts are involved in the focus modulation, scanning is achieved without hysteresis [18]. Notably, the control of the electrical voltage amplitude applied to the TAG lens enables one to select the range of focal lengths and, consequently, the axial scanning range.

One of the main benefits of high-speed z-scanning confocal microscopy is presented in Fig. 1(b). In this case, an image is captured with the TAG lens on and the detector signal is integrated along the pixel-dwell time. Since the signal-dynamic during the pixel-dwell time is ignored, a conventional acquisition card can be used for this purpose. The high z-scanning speed of the system allows to perform a full sweep of the focal position in a short time (~7 µs). However, to reach a suitable signal-to-noise ratio and to match the minimum pixel-dwell time of the microscope several axial scans are performed during each pixel cycle. As a result, photons corresponding to different focal positions are collected within the same pixel obtaining an image with an EDOF. The tunability of the system is such that the degree of depth of field enhancement can be controlled by simply adjusting the TAG lens voltage amplitude. Similar approaches have been reported for two-photon microscopy [20] and for laser processing [21], but high-speed z-scanning EDOF has never been used in confocal microscopy. In this latter case, the possibility of user-controlled EDOF opens the door to the study of fast dynamic processes [7] in fields as important as flow cytometry [22]. Despite the possibility to survey an entire 3D specimen at a glance, EDOF confocal microscopy sacrifices

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important information concerning the z-position of the collected photons. This can be a limiting factor in several applications requiring accurate depth discrimination.

The inherent problem of EDOF can be solved by using a fast (>>140 kHz) acquisition card properly synchronized with the TAG lens scanning [Fig. 1(c)]. In this way, one can gather information of the photon arrival time with respect to the lens oscillation and determine the corresponding focal plane on a pixel by pixel basis. Since the photons can be grouped into different time intervals, multiple focal planes can be retrieved with a user-selectable optical thickness. Accordingly, in a single XY scan multiple focal planes can be simultaneously imaged, enabling high-speed 3D confocal microscopy. One can consider high-speed z-scanning as a simple spatiotemporal multiplexing method in which a fast acquisition card is needed for accurate demultiplex readout. Notably, this method greatly simplifies traditional spatiotemporal multiplexing for simultaneous multiplane imaging based on splitting and delaying the excitation beam by using several lenses and mirrors [23]. Indeed, the inherent complexity of the traditional approach has limited its applicability to multiphoton microscopy [24] where only the excitation pathway requires multiplexing. High-speed z-scanning circumvents these limitations allowing multiplexing of both excitation and detection pathways as required in confocal methods, opening the door to simultaneous multiplane confocal microscopy.

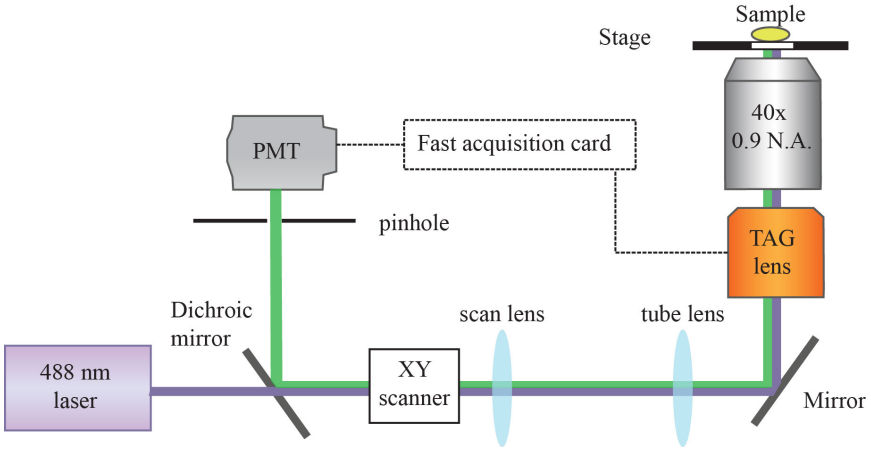

Fig. 2. Scheme of the high-speed z-scanning confocal microscope. The acoustically driven optofluidic lens (TAG lens) is placed at the back focal aperture of the objective of a commercial microscope, enabling light modulation in both excitation and detection paths.

In this particular experiment, we use a commercial confocal microscope (Fluoview 1000, Olympus) equipped with a water immersion objective lens (Olympus WLSC, 40x, NA 0.9), a XY galvo mirror scanner and a motorized z-stepper (10 nm resolution) normally used for slow 3D imaging. High-speed z-scanning is enabled by a TAG lens 2.0 (TAG Optics Inc.) placed at the back focal plane of the objective lens (Fig. 2) and whose piezoelectric cylinder (16 mm in diameter, 20 mm long) is driven at a frequency of 143 kHz and at a voltage amplitude up to 20 Vpp. The TAG lens can operate at resonant frequencies ranging from 70 kHz to 1 MHz, but the effective lens aperture decreases with the driving frequency. At 143 kHz there is the optimal tradeoff between TAG lens aperture relative to the objective lens used, and speed. The location of the TAG lens at the back focal plane of the objective lens or in a conjugate plane has been proved optimal for achieving the highest scanning range and minimizing vignetting [19]. In addition, it assures that the lens is in both excitation and detection pathways. This requisite is essential in confocal microscopy in order to conjugate the excitation focal plane with the pinhole plane and thereby preserve the confocal sectioning capability. Due to the compact size of the TAG lens, the integration of the lens into the commercial confocal system only requires lifting both objective and stage about 30 mm from its original position, resulting in minimum and low-invasive system changes. We acquire

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images using a fast acquisition card (DPC-230, Becker and Hickl GmbH, ~180 ps time resolution) synchronized with the XY scanner of the microscope (pixel, line and frame clocks) and with the TAG lens oscillation. We use the acquisition card in a time-tagged modality. Notably, this obviates the need to mutually synchronize TAG lens and XY scanner. In order to detect the same number of photons at each focal plane, the pixel clock should be selected to be an integer multiple of the TAG lens oscillation (non- integer values can be used as well but some collected photons need to be discarded during the photons grouping). However, this requirement can be minimized or even obviated if several axial scans are performed at each pixel, as it occurs in this current experiment. In all experiments, the pinhole size is 1 airy unit and the operating excitation wavelength is set at 488 nm. Fluorescence emission is collected in the 500-560 nm range.

3. Results

3.1 Microscope characterization

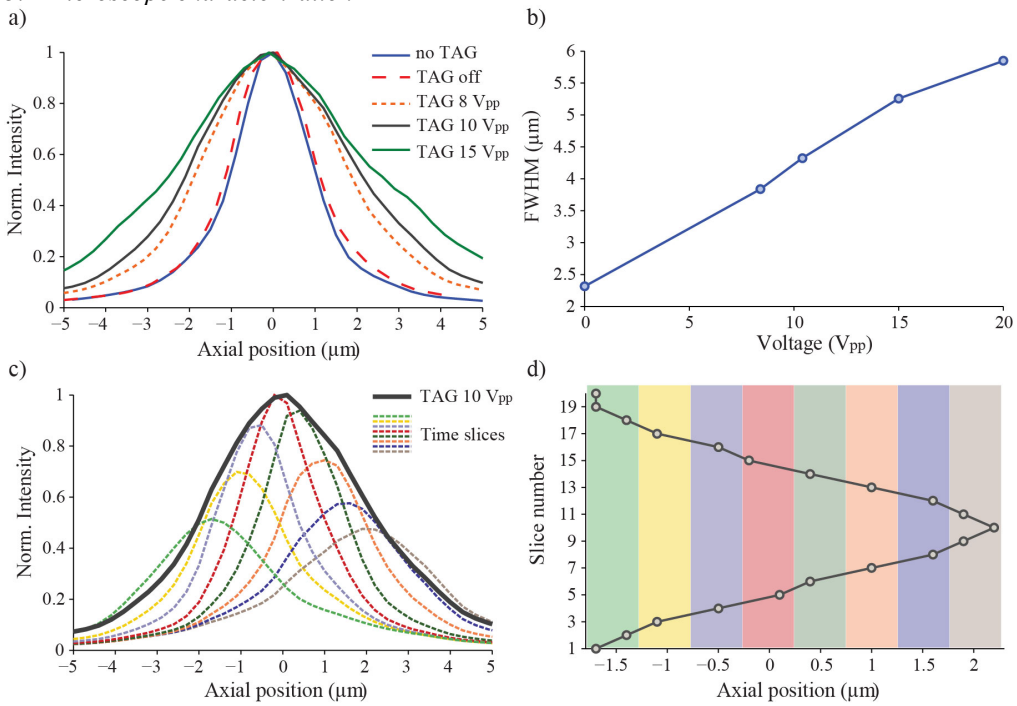

Fig. 3. Optical performance of the high-speed z-scanning confocal microscope. a) Plot of the normalized intensity of a thin fluorescence sample at different axial positions for different driving voltages. By changing the voltage amplitude applied to the TAG lens, the full width at half maximum (FWHM) of the axial intensity can be modified, demonstrating a user-controllable EDOF. b) Plot of the FWHM versus TAG lens driving voltage. c) Multi-plane imaging by spatiotemporal multiplexing. The information of the photons arrival time with respect to the TAG lens oscillation is used to group the collected photons (thick line) into arbitrarily defined time intervals (thin lines). Such intervals correspond to different slices or focal planes. d) Plot of the different slices with respect to their axial position. Since the TAG lens produces a sinusoidal scanning, we first select 20 time intervals (dots) that we then regroup according to their axial position into 8 different slices (colored bars) in order to enhance the SNR.

We first characterize the EDOF capabilities of our high-speed z-scanning confocal microscope by imaging an homogeneous thin layered fluorescent sample (110 nm thick) at different axial distances from the objective lens each separated by 300 nm using a programmable stepper motor. The full-width at half-maximum (FWHM) of the fluorescence signal along the axial direction, corresponding to the system depth of field (DOF), is presented in Fig. 3(a) for different conditions. Notably, placing the TAG lens in the

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microscope does not significantly affect the optical sectioning performance of the system, with just an increase in the system FWHM of less than 5%. As we turn the TAG lens on, the system FWHM increases extending up to 6 μm for 20 Vpp, more than a factor of 3 longer than the system initial DOF. In addition, the enhancement in DOF is approximately linear with the TAG applied voltage amplitude [Fig. 3(b)], which facilitates the selection of a particular axial range of interest.

Simultaneous multiplane imaging is enabled by using a fast acquisition card adequately synchronized with the TAG lens modulation. An initial calibration is required to establish the relationship between photon arrival time and focal plane. We perform such calibration by translating and imaging the thin fluorescence sample at predefined positions (300 nm steps) by means of the motorized stage [Fig. 3(c)]. In this particular experiment, we drive the TAG lens at 10 Vpp. Obviously, the sum of all collected photons [Fig. 3(c), thick line] results in the same EDOF obtained in Fig. 4(a). However, in this case the additional temporal information of the photons arrival enables one to group the collected photons into different time intervals with user-defined durations. In this experiment we select 20 time intervals per cycle, each with a duration of about 350 ns, corresponding to 1/20 of the TAG lens oscillation period (~7 μs). A plot of 8 of this time intervals is presented in Fig. 3(c) (thin lines). Notably, each of these intervals corresponds to a different portion of the optical axis, i.e. to a different focal plane. As we move further from the initial objective focal plane (position 0 μm), the maximum intensity of the optical sections decreases and their FWHM increases. This degradation in axial performance is attributed to aberrations induced by changes in the effective NA of the objective as the TAG lens oscillates, but could be compensated by placing another TAG lens in front of the confocal pinhole [14]. The plot of the axial position for the 20 slices presented in Fig. 3(d) reveals that the total scanning range is about 4 μm. Since the TAG lens creates a sinusoidal translation of the focus [21], the axial spacing between slices is not constant, but it decreases as the slices reach the scan limits. As a result, we can group the different slices according to their axial position [colored bars in Fig. 3(d)]. This has the added advantages of increasing the signal to noise ratio (SNR) per optical section and to compensate for the loss of intensity that occurs at the edges of the scanning range. In this experiment, sorting the slices into 8 different groups, each with an axial extent of about 500 nm, provides the optimum SNR and image quality.

We can analyze the speed capabilities of our multiplane confocal system by considering the number of photons ($n_{photons}$) collected by the detector during image acquisition to be given by:

$$n_{photons} \propto I \times time_{pixel} \times n_{pixel_x} \times n_{pixel_y} \tag{1}$$

where n_{pixel} represents the number of pixels in the x and y directions, $time_{pixel}$ is the pixel dwell time, and I is light intensity (number of photons per unit area and unit time). As we axially scan the focus, the number of photons collected at each focal plane decreases linearly with the number of planes selected. We can compensate for this loss in signal by simply increasing the pixel dwell time. This is an effective method that slightly sacrifices temporal resolution but still results in acquisition speeds much higher than conventional z-scanners since it obviates the need for mechanical translation, acceleration and repositioning time. Alternatively, one can increase light intensity. For optical metrology applications, this is a valid method that can be usually implemented without risk of photodamage. In the case of fluorescence, high intensities can potentially lead to photobleaching. It is also worth noting that high-speed z-scanning requires fluorophores with fluorescence lifetimes shorter than the voxel dwell time. This is not a strong constraint since most organic fluorophores and fluorescent proteins have lifetimes in the order of nanoseconds, more than one order of magnitude faster than required by the TAG lens (350 ns). In any case, the ultimate speed limitation of our approach is not inertia as it occurs with traditional z-scanners or other electronic tunable lenses, but it is given by the ability for photon collection or, equivalently, the SNR.

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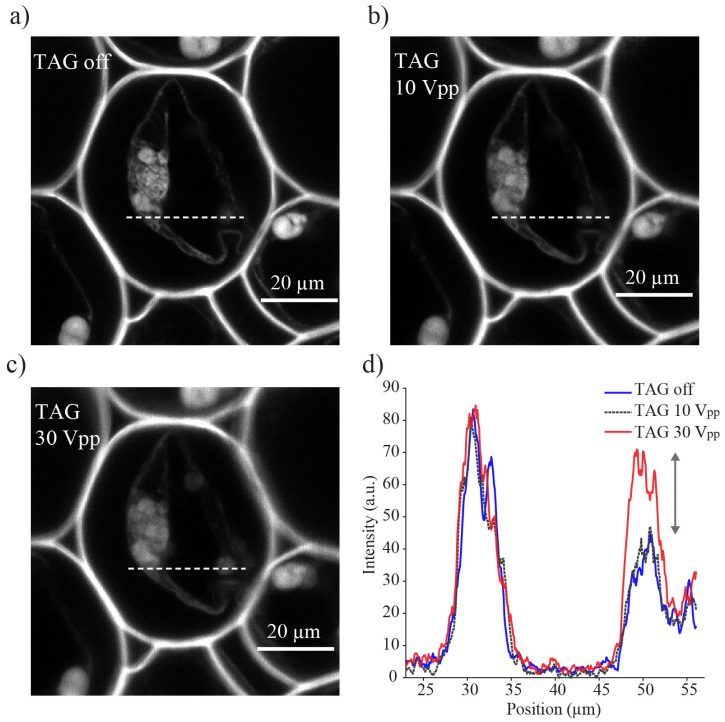

Fig. 4. EDOF confocal microscopy of a Convallaria majalis sample. a) When the TAG lens is off, only an optical section is captured. b-c) As the voltage applied to the TAG lens increases, the DOF increases. In this way, details that were initially at different focal planes and thereby remained hidden, become visible. d) Plot of the intensity across the dotted line in Figs. 3(a)–3(c). The arrow indicates the increase in intensity for EDOF microscopy for an object initially situated out of focus.

3.2 Imaging biological samples

The use of high-speed z-scanning EDOF confocal microscopy is presented in Fig. 4 for an autofluorescent biological sample (Convallaria majalis). By increasing the TAG lens voltage amplitude, objects that were initially out of focus and remained hidden start to become visible. The comparison of the intensities across a line situated at identical locations for the three images [Figs. 4(a)–4(c)] clearly reveals the relative gain in intensity of initially hidden objects [Fig. 4(d)], demonstrating a user-controlled enhancement in DOF. Contrary to other existing methods such as remote focusing [7] or wavefront encoding [25], this approach to EDOF microscopy does not require complex optics and can be easily integrated in different systems or even in other confocal modalities, offering a promising way to study the dynamic behavior of 3D specimens at a high-temporal resolution.

We use the same Convallaria sample to compare the proposed simultaneous multiplane imaging method with the conventional axial scanning method based on the mechanical movement of the sample (or similarly of the objective lens) (Fig. 5). A conventional 2D XY confocal image obtained by scanning the laser beam in the XY directions using galvanometric mirrors is presented in Fig. 5(a). Notably, by using the TAG lens one can simultaneously capture 8 focal planes in a single XY scan [Fig. 5(b)]. In this experiment, to maintain SNR we increase the pixel time by a factor of 3 and increase the excitation intensity by another factor of 3. The good agreement between the images obtained with TAG-enabled confocal microscopy and those using a traditional mechanical z-stage is shown in Fig. 5(c). It is important to note the complete different approach used to capture these two sets of images. Whereas traditional z-scanning requires stepwise translation of the focal plane between images, the TAG-enabled microscope captures all images simultaneously by continuously z-

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scanning the beam at each pixel. To quantify the agreement between the two 3D methods we calculate the cross-correlation coefficient of the corresponding images. The values obtained with the TAG lens, in the range of 0.91 and 0.98 [Fig. 5(d)], are significantly larger than those corresponding to the decorrelation of the sample with depth. In addition, the cross-correlation coefficient deteriorates as one moves away from the initial focal plane. This can be attributed to the dependence of this parameter on effects such as rotation, translation, luminance fluctuations or even changes in magnification that may occur during fast z-scanning. In any case, the values of Fig. 5(d) confirm that high-speed z-scanning enables one to obtain images very similar to those obtained using traditional confocal microscopy. Finally, by using 3D rendering algorithms [26] we can combine the images captured with the TAG-enabled microscope to obtain a 3D image of the biological sample [Fig. 5(e) and Media 1], which proves the feasibility of high-speed z-scanning confocal microscopy for 3D imaging.

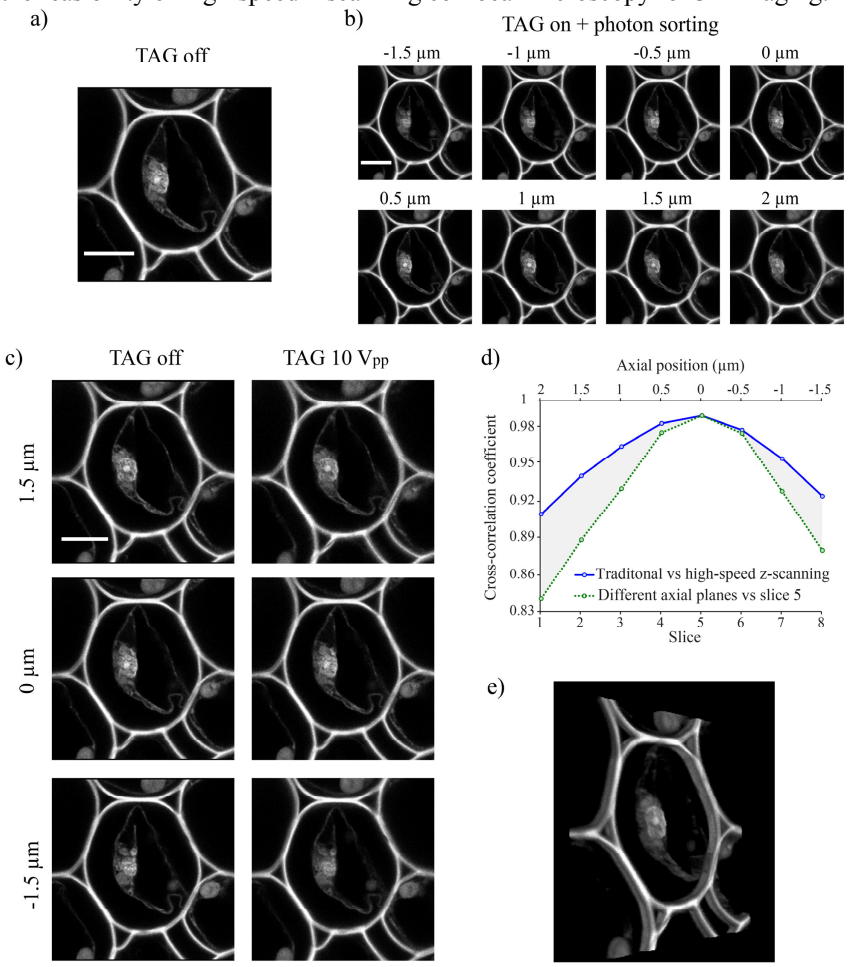

Fig. 5. Multiplane imaging confocal microscopy of a Convallaria majalis sample. a) Conventional confocal image of a thin optical section of a biological sample. This image is obtained by mechanically scanning the laser beam in the XY plane using galvanometric mirrors. b) By high-speed z-scanning confocal microscopy (TAG lens at 10 Vpp) and appropriate time sorting, a single XY scan results in 8 different images each corresponding to a different focal plane. c) Comparison of the images obtained with a traditional z-stage and those of high-speed z-scanning confocal microscopy. d) Cross-correlation coefficient of the images obtained using the two methods. A comparison between the different axial planes and slice 5 is also included as a reference to show how rapidly the structure decorrelates with depth. The difference between both curves has been highlighted in gray. e) Video frame of the 3D image reconstructed using the high-speed z-scanning confocal microscope (Media 1). Scale bars are 20 µm.

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4. Conclusion

The high-speed z-scanning confocal microscope presented in this article offers a promising approach toward high-speed 3D confocal microscopy. Our microscope is based on an optofluidic device that produces axial scanning speeds of 140 kHz without mechanical moving parts. Such high-speed enables one to capture photons from different focal planes on a pixel by pixel basis. The integration of all photons collected at each pixel produces an image with an EDOF whose extension can be user-controlled. By using synchronized and high time-resolution detection, the collected photons can be grouped into different time intervals each corresponding to a different focal plane. This allows the simultaneous acquisition of multiple focal planes with an image quality comparable to that of traditional z-scanning methods. Since this novel approach for fast 3D confocal microscopy obviates mechanical scanning, the only speed limitation is due to the SNR. As the sensitivity of photodetectors increases and more efficient fluorescent labeling is developed, our approach could be used to unveil the dynamics of 3D processes that occur on the submillisecond time scale.

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