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Abstract. Traditional white-light and fluorescent imaging techniques provide powerful methods to extract high-resolution information from two-dimensional (2-D) sections, but to retrieve information from a three-dimensional (3-D) volume they require relatively slow scanning methods that result in increased acquisition time. Using an ultra-high speed liquid lens, we circumvent this problem by simultaneously acquiring images from multiple focal planes. We demonstrate this method by imaging microparticles and cells flowing in 3-D microfluidic channels. © *2012 Society of Photo-Optical Instrumentation Engineers (SPIE).* [DOI: 10.1117/1.JBO.17.5.050505]

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One of the key frontiers in modern imaging is to extract real-time three-dimensional (3-D) images from a living biological system or fast moving industrial process.[1] A promising way to achieve this is to acquire information from multiple locations in a sample as quickly as possible, ideally simultaneously. Previous approaches based on this concept include spatiotemporal multiplexing in two-photon microscopy,[2–4] remote focusing in confocal microscopy,[5] or modifying the detection pathway of a conventional microscope to send information from two different depths into two separate cameras.[6,7] However, requirements for mechanical scanning limit the acquisition speed in the first two classes of techniques, whereas the latter techniques lack flexibility in selecting the image plane locations due to the fixed position of the cameras and optics.

Here, we propose a novel method for simultaneously acquiring multiple and selectable focal planes without mechanically moving parts. Our approach is based on an acoustically driven liquid lens which is used to scan through different axial planes at frequencies as high as 1 MHz. Such high speed, in combination with synchronized pulsed illumination, enables one to select any desired focal plane within the lens scanning range. We use multiple light sources at different wavelengths, each distinctly synchronized with the lens to record information corresponding to different locations within the volume in a single exposure of a standard color charge-coupled device (CCD) microscope camera. Although the composite color image merges the different planes, simply by separating the color channels of the image, we can recover each individual focal plane without loss of in-plane lateral resolution. We demonstrate our method by imaging polystyrene microparticles and living cells flowing through 3-D channels from a microfluidic chip.

The unique imaging capabilities of our method are enabled by the use of a tunable acoustic gradient index (TAG) lens.[8] This device consists of a fluid-filled cylindrical cavity which is radially excited with acoustic energy causing a periodic standing wave modulation in the fluid, and consequently, the index of refraction.[9] As such, the lens is continuously varying its focal length and therefore, by defining a time delay between the acoustic field and the incident light any specific focal length can be selected with submicrosecond temporal resolution [Fig. 1(b)]. The feasibility of the TAG as a varifocal device has been previously demonstrated,[8,10] but the high-speed focusing capabilities have never been used to simultaneously acquire multiple images from different focal planes. In this particular experiment, we drive a TAG lens made of a cylindrical piezoelectric, inner diameter of 32 mm and length of 40 mm, with a USB function generator (Syscomp, WGM-201) at a frequency of 72 kHz. We set up the TAG lens on top of the objective revolver of a transmission bright field microscope (Olympus BX 60), as ray tracing simulations show that this location is optimal for achieving the highest scanning range as well as maximizing the exit pupil. We employ two color pulsed illumination to select two focal planes for simultaneous imaging, and we acquire images using a CCD color camera (Lumenera, Infinity 1) located after the microscope tube lens [Fig. 1(a)].

We first determine the scanning capabilities and resolution of the TAG-enabled transmission microscope. In this case, we use a pulsed white LED to illuminate a calibration sample which consists of a tilted ruler. Varying the time delay between light pulses and the TAG with a pulsed delay generator (Stanford Research System DG535) allows the user to select an arbitrary focal plane within the sample. This can be observed in Fig. 1(c), where different parts of the ruler appear in focus at different delay times (α, β, γ) with the corresponding height profile of this sample displayed at the bottom of Fig. 1(c). In this particular experiment, the scanning range (Δz) of the TAG-enabled microscope for a 10× objective (N.A. 0.25) is about 300 μm, but this range depends on the objective magnification. We model Δz for different infinity-corrected objectives using geometrical optics and thin lens approximation. Accordingly, we consider all the elements in the microscope, including objective, TAG and tube lens, as thin lenses with a known optical power δ and location. The thin lens equations for this system can be written as:

$$1/s + 1/s' = \delta_{\text{objective}} \tag{1}$$

$$1/(d - s') + 1/s_2' = \pm\delta_{\text{TAG}}, \tag{2}$$

where s is the distance between the object and the objective, s' is the distance between the image after the object and the

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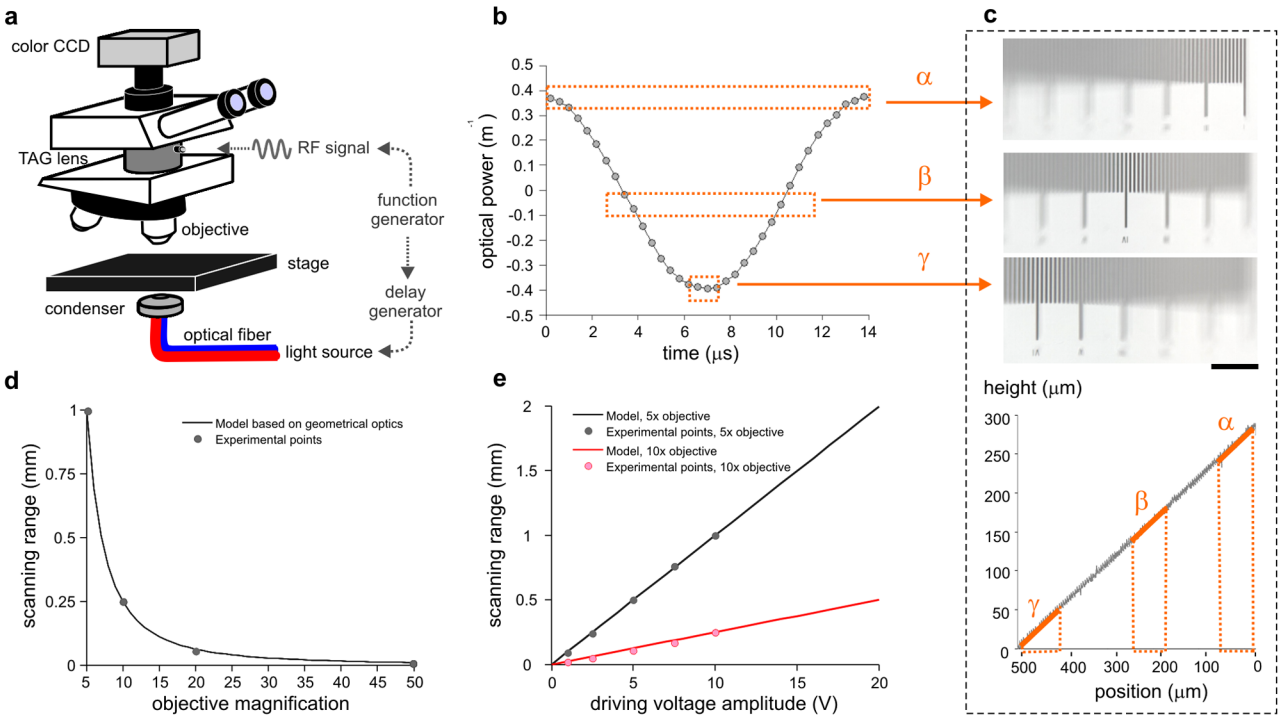

Fig. 1 TAG-enabled microscope setup and characterization. (a) Schematic representation of the setup used in the experiments. (b) Temporal evolution of the TAG optical power when driven at a frequency of 72 kHz. The TAG is in a continuous state of changing focus. (c) Images of a tilted ruler acquired using pulsed light synchronized with the TAG at different delay times (α, β, γ) and the corresponding ruler profile at the bottom. Scale bar, 100 μm. (d) Scanning range of the microscope versus different objective magnifications: 5x (N.A. 0.13), 10x (N.A. 0.25), 20x (N.A. 0.40), and 50x (N.A. 0.50). The dot points correspond to the experimental values range of the microscope scales linearly with the voltage amplitude of the signal that measured, and the solid line to the predictions of a model based on geometrical optics. (e) The scanning drives the TAG lens. This is due to the linear dependence between TAG optical power and driving amplitude.[8] The dot points correspond to experimental measurements and the solid lines to the predictions based on a geometrical optics model.

objective, s_2' is the distance between the image after the TAG lens and the TAG lens, and d is the separation between the objective and the TAG lens. Since the camera is located at the focus of the tube lens, $s_2' = \infty$. The microscope scanning range is then determined by the solution of the previous equations over the change in focal power of the TAG from δ_{TAG} to $-\delta_{\mathrm{TAG}}$:

$$\Delta z = |s(\delta_{\mathrm{TAG}}) - s(-\delta_{\mathrm{TAG}})| = \frac{2f_{\mathrm{tube}}^2 \delta_{\mathrm{TAG}}}{M^2 - \delta_{\mathrm{TAG}}^2 (Md - f_{\mathrm{tube}})^2}, \tag{3}$$

where $\delta_{\mathrm{objective}} = M/f_{\mathrm{tube}}$ has been considered, M being the objective magnification and f_{tube} the focal length of the tube lens. This model is in good agreement with experimental results [Fig. 1(d)]. Notably, Δz can be easily controlled by its linear relationship to the amplitude of the driving signal [Fig. 1(e)]. In this way, it is possible to achieve a Δz in all cases that is superior to the objective depth of field (DOF) by more than an order of magnitude. In addition, and given the analog nature of the TAG, it is possible to continuously select between the different focal positions within that range.

The use of TAG-enabled microscopy for simultaneous imaging of multiple focal planes is presented in Fig. 2 for a PDMS microfluidic chip containing polystyrene microbeads. In this case, we use a red and blue pulsed LED to illuminate the beads flowing through two microchannels located 1 mm apart from each other in the z direction [Fig. 2(b)]. A pulse delay generator drives each light-emitting diode (LED) (light pulse duration of 600 ns) synchronously with the TAG, but each with a different delay time in order to select the desired focal plane and we capture both pulses within a single exposure of a color CCD camera [Fig. 2(c)]. By separating the colors of the image from the CCD output, we recover information of the entire field of view of each individual plane. Furthermore, the separated image does not show color leakage due to the monochromacity of the LED sources. This process can be scaled up with additional illumination colors.

We also evaluate real-time imaging of simultaneous multiple planes with the TAG-enabled microscope. In this case, we show a video of microspheres flowing through the two channels of the microchip in Fig. 2(b) at 8 frames per second (fps) (Video 1). The video shows how the microspheres move at different speeds and directions as we manually vary the flux in each individual channel. In this case, the limitation to the acquisition time is the frame rate of the camera itself. However, given a bright illumination source, the theoretical temporal resolution of the TAG-enabled microscope is given by the time required to scan over Δz, which in this case is 14 μs or equivalently 72,000 fps. This value is orders of magnitude higher than the maximum speed that can be obtained by mechanical scanning systems which are subject to settling times and have to overcome inertial effects.

Finally, we explore the potential of the TAG-enabled microscope for imaging biological systems (Fig. 3). In this case, murine fibroblast cells flowing through two perpendicular microchannels separated by 300 μm are imaged in a single exposure of a color CCD camera, applying the same method described above [Fig. 3(a)]. The color separation of the CCD image allows

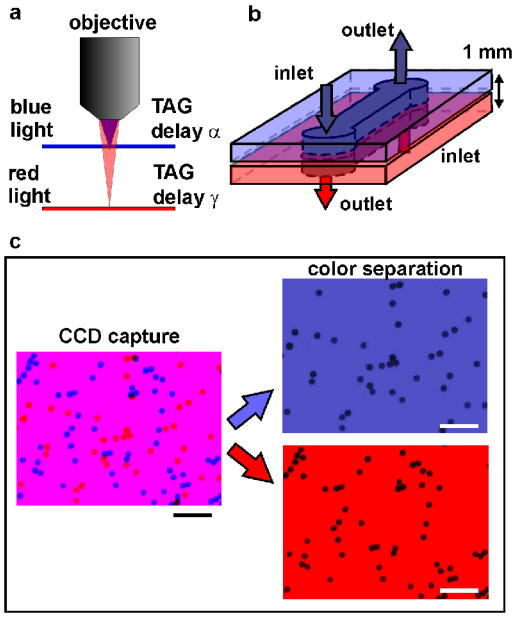

Fig. 2 Simultaneous imaging of multiple focal planes by a TAG-enabled microscope. (a) Scheme of the operation principle of the microscope, in which light pulses of different colors corresponding to different focal planes when synchronized with the TAG, are simultaneously acquired in a single CCD camera exposure. (b) Representation of the microfluidic device consisting of two microchannels separated by 1 mm through which polystyrene microspheres (diameter of 15 μm) flow. (c) On the left, composite color CCD image with the spheres corresponding to each microchannel merged. On the right, color separation of the image allows recovering information of the spheres in each individual microchannel without loss of in-plane lateral resolution (Video 1, MOV, 3 MB). Scale bars, 100 μm. [URL: http://dx.doi.org/10.1117/1.JBO.17.5.XXXXXX.1]

recovering information of the cells in each individual microchannel without loss of in-plane information [Fig. 3(b) and 3(c)]. In addition, by using focus stacking algorithms[11] we are able to generate a two-dimensional image of the cells flowing in each microchannel where out of focus information has been eliminated [Fig. 3(d)].

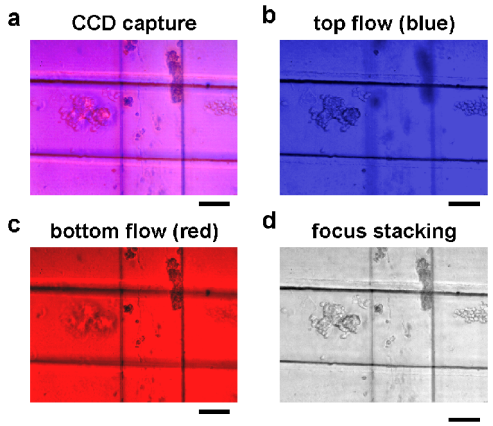

Fig. 3 Simultaneous imaging of murine fibroblast cells flowing through two perperndicular microchannels separated by 300 μm. (a) Composite color CCD image acquired in a single exposure. (b,c) Color separation of the image in (a) provides information of the cells flowing in each individual microchannel. (d) Focus stacking of the images from (a) into a single image where out of focus information can now be removed. Scale bars, 100 μm.

The possibility of simultaneously imaging multiple focal planes demonstrated in this manuscript for optical microscopy is a first step toward real-time 3-D microscopy. Our method does not require mechanically moving parts and it can be scaled up by using multiple colors, which opens the door to record multiple focal planes at speeds as high as 72,000 fps. In the future, the implementation of imaging processing algorithms will make it possible to retrieve the phase information of the images which can enable the reconstruction of the 3-D space between the corresponding focal planes.[12] In addition, applying our method in combination with other imaging techniques based on optical sectioning such as two-photon microscopy,[13] confocal microscopy,[14] or structured illumination methods,[15] can result in a significant reduction of the acquisition times allowing researchers to explore new fundamental processes in living systems.

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