Remote focusing for programmable multi-layer differential multiphoton microscopy

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Abstract: We present the application of remote focusing to multiphoton laser scanning microscopy and utilize this technology to demonstrate simultaneous, programmable multi-layer imaging. Remote focusing is used to independently control the axial location of multiple focal planes that can be simultaneously imaged with single element detection. This facilitates volumetric multiphoton imaging in scattering specimens and can be practically scaled to a large number of focal planes. Further, it is demonstrated that the remote focusing control can be synchronized with the lateral scan directions, enabling imaging in orthogonal scan planes.

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1. Introduction

The ability to image within scattering media has been greatly enhanced by the introduction of laser scanning microscopes. In particular, the ability to produce two photon excited fluorescence (TPEF), second harmonic generation (SHG), and third harmonic generation (THG) using these microscopes permits researchers to perform unique internal explorations deep within biological systems [1].

Multi-focal laser scanning microscopes were introduced to improve the image acquisition time of laser scanning microscopes. These systems improve the speed of traditional laser scanning microscopes through the application of additional foci within the sample specimen. There are several methods for approaching the generation of these foci, with various advantages and disadvantages. Common approaches to the problem involve the use of micro-lens arrays [2–8], etalons [9], diffractive optical elements [10], or cascading beam splitters [11–15].

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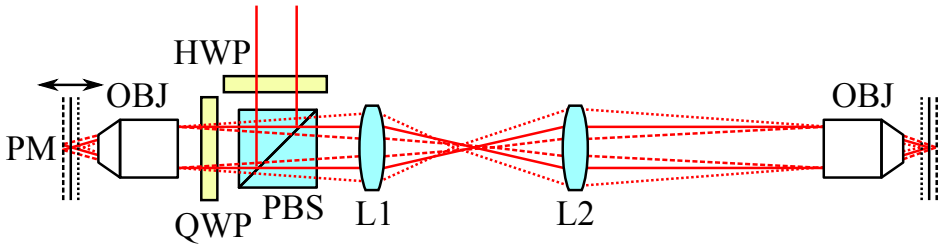

Fig. 1. Basic axial scanning microscope system. HWP: Half Wave-plate, PBS: Polarizing Beam-splitter, QWP: Quarter Wave-plate, PM: Piezoelectric Mirror, L1,L2: Matched Lenses, OBJ: Objective, Solid Beam: Normal (collimated) focus, Dotted Beam: Shallower focus, Dashed Beam: Deeper focus

Since these techniques utilize closely spaced or overlapping beams, it is important to use a spatially-resolved detector and temporally separate the arrival time of the beams in order to avoid interference of the excitation beams [4–6, 12]. Interference reduces the axial sectioning, and utilizing a spatially resolved detector diminishes the ability to image within scattering specimens.

We previously reported on an alternative method for approaching multi-focal foci generation wherein a special laser cavity is employed that outputs beams that are both temporally and spatially separated [16]. Since this cavity design delays the beams by several nanoseconds, it becomes possible to use a single-element detector, thereby taking advantage of the speed improvements of a multi-beam system without sacrificing image quality through the use of a spatially-resolved detector. When compared to other multi-focal techniques, this system is also advantageous in that the beams are spatially separated by several millimeters, allowing for manipulation of the individual beams with little difficulty. These characteristics produce an extremely flexible imaging system that is capable of *simultaneously* imaging specimens at multiple different layers [17], employing different excitation polarizations [18, 19], or different pulse shapes [20, 21]. As a result of the myriad of possible applications for this imaging technology, we refer to the general approach as “differential multiphoton microscopy”.

While all of the different multi-focal systems produce demonstrable improvements to data acquisition in the sample plane, they still do not fully resolve the issue of imaging biological systems in three dimensions. Since the primary focus of these laser systems is to image within living organisms, it is crucial to record data from more than one sample plane in order to get an accurate picture of what is occurring within the organism. Typically, this problem is resolved by alternating between scanning and moving the sample or the objective in order to acquire an image from another plane. However, the rate of image acquisition at different depths can be further optimized through the technique of remote focusing, or changing the beam divergence to change the sample depth, introduced by Botcherby et al. [22–25]. Our multi-beam cavity design is well suited to exploit this technique, as we can readily adjust the focal depth of each excitation beam and therefore acquire images from multiple planes simultaneously.

In this work we demonstrate the viability of remote focusing by using 1) a dual focus multiphoton differential laser scanning microscope, and 2) in conjunction with our novel multi-beam oscillator [16], a four beam imaging system. To our knowledge, this is the first multifocal laser scanning microscope architecture that can simultaneously image multiple axially separated focal planes that are under electronic control through remote focusing.

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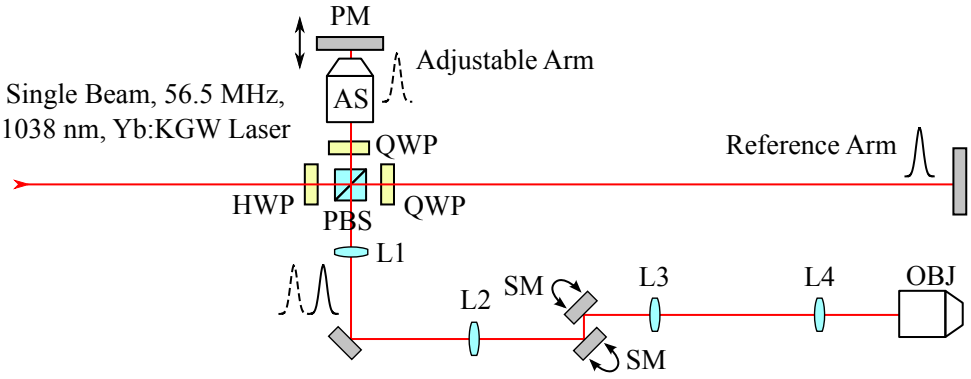

Fig. 2. Two beam remote focusing microscope layout. HWP: Half Wave-plate, PBS: Polarizing Beam-splitter, QWP: Quarter Wave-plate, AS: Asphere, PM: Piezoelectric Mirror, L1: 250 mm Lens, L2: 500 mm Lens, SM: Scan Mirror, L3: 200 mm Lens, L4: 200 mm Lens, OBJ: Objective

2. Dual focus differential laser scanning microscope system

2.1. Remotely controlling axial focus with a piezoelectric mirror

By adjusting the axial focus through changing the properties of the beam, rather than mechanically moving the objective or the sample, remote focusing facilitates volumetric imaging of living organisms. A similar imaging improvement was introduced to confocal laser scanning microscopy with the introduction of galvanometric scanners by Åslund et al. [26].

Whereas the improvement to scanning of the focal plane was achieved by changing the angle of the collimated light entering the back of the objective, the key to adjusting the position of the axial focus is to change the divergence of the beam at the back of the microscope objective. As demonstrated for axial scanning by Botcherby et al. [24], this scenario is most easily accomplished by imaging the plane at the back of the microscope objective to a second identical objective with a 1:1 telescope (Fig. 1). By focusing the light of this "adjustment objective" onto a mirror attached to a piezoelectric actuator, the position of the axial focus of the imaging objective can thereby be programmatically controlled.

2.2. Experimental setup

Our dual focus scan system is shown in Fig. 2. The input to this system is a Yb:KGW laser that operates at a central wavelength of 1038 nm, a repetition rate of 56.5 MHz, produces 2.5 W average output power (45 nJ/pulse) and pulses ~250 fs in duration [19]. The remote focusing arm of this system uses a 0.65 NA aspheric lens (New Focus 5721-H-B) focused onto a zero-degree high reflector mirror attached to a piezoelectric actuator (Thorlabs PAS080). The piezoelectric actuator is then driven by an open-loop piezo controller (Thorlabs MDT694A) connected to a PC with a DAQ card (National Instruments PCI-6259) in order to control the axial focal position. The second focus is shown as the reference arm, and is delayed by approximately 8.8 ns (approximately half the repetition period of our 56 MHz system) relative to the remote focusing arm. The second focus can act as a control. For example, when the piezo is at the "zero" position it is used to ensure that the optics in the adjustable arm are not adding significant aberrations to the remote focusing beam. As previously shown, we can image with both arms simultaneously [19]. This configuration enables imaging at different depths, at different excitation polarizations, and in different modalities.

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To satisfy the imaging constraints imposed by remote focusing, while still permitting lateral scanning, a couple of steps are involved. First, the plane at the back of the New Focus asphere is imaged with a 1:2 telescope to the telecentric plane between the galvanometric scan mirrors (GSI Group, Inc SC2000). A 1:2 telescope was chosen for this configuration in order to expand the laser beam to better fill the back of the microscope objective (Zeiss A-Plan 40x/0.65). This choice allows for more effective use of the numerical aperture of the objective at the cost of half of the axial movement range. However, since movement of the piezo mirror actually results in twice that movement at the sample plane, this sacrifice places us in a regime where the movement is approximately 1:1 (80 μm for the PAS080 actuator). In other words, the spatial shift of the mirror on the piezoelectric mount is the same as the axial shift of the focus. The second step is to image the telecentric plane between the scan mirrors using a 1:1 telescope to the back of the microscope objective. This system is integrated with an Olympus IX71 microscope platform, so the second lens of the telescope (L4) is the tube lens of the microscope platform. While this system is slightly more complicated than a traditional laser scanning microscope, in exchange for this complexity we gain control over the axial position of the beam without having to move either the sample or the objective.

While this piezo setup can be used in a stand-alone fashion to select desired *x*-*y* imaging planes, it can also be synchronized with the scan mirror hardware in order to image non-standard geometries, such as the *x*-*z* plane. To demonstrate this capability, we created a program for our scan mirror hardware to use an external digital synchronization signal to trigger each *x*-axis scan, rather than to trigger each *x*-axis scan after each step of the *y*-axis. This trigger is then driven by the DAQ card on our PC, permitting us to simultaneously control both the piezo and the scan mirrors.

2.3. Consequences of utilizing an aspheric lens

An aspheric lens is used throughout this work. The primary motivation being that for a multi-beam system the cost of implementing the remote focusing is significantly reduced. However, aspheres do exhibit axial color. For the Yb:KGW laser design used here, ray-tracing models indicate an axial offset that is below the diffraction limit over our maximum laser bandwidth of 6 nm (centered at 1038 nm).

2.4. Imaging results

In order to demonstrate the axial characteristics of this system we modified our traditional *x*-*y* scanning application to permit *x*-*z* scanning while leaving the sample stationary. In other words, we have synchronized the remote focusing with a single lateral scan axis (the x-axis), enabling us to rapidly capture image planes that are orthogonal to the traditional lateral image plane. To demonstrate this capability we use third harmonic generation (THG) imaging of complex structures fabricated in a 500 micrometer thick fused quartz slide through spatio-temporal focused femtosecond laser micromachining [27–29]. These structures are produced when the fabrication scan direction is coincident with the traveling wave created by pulse front tilt, which is inherent to machining with spatio-temporal focused femtosecond laser pulses [29].

An *x*-*y* THG image slice and an *x*-*z* THG image slice of the structures is shown in Fig. 3, with a corresponding *x*-*y* white light image of the region in the inset. The *x*-*z* image slice clearly reveals the axial extent of the structure, and locates the structure relative to the quartz surface, as evidenced by the image of the quartz-air boundary at the bottom of the *x*-*z* scan. The *x*-*z* image demonstrates the typical axial scan range achieved in the dual focus system, which is effectively 80 micrometers. We characterized the focus over the scan range using the axial THG response at an interface and found that the full width at half maximum differs from the diffraction limited value of 6.9 micrometers (for 0.65 NA, 1039 nm) by at most 8% at the

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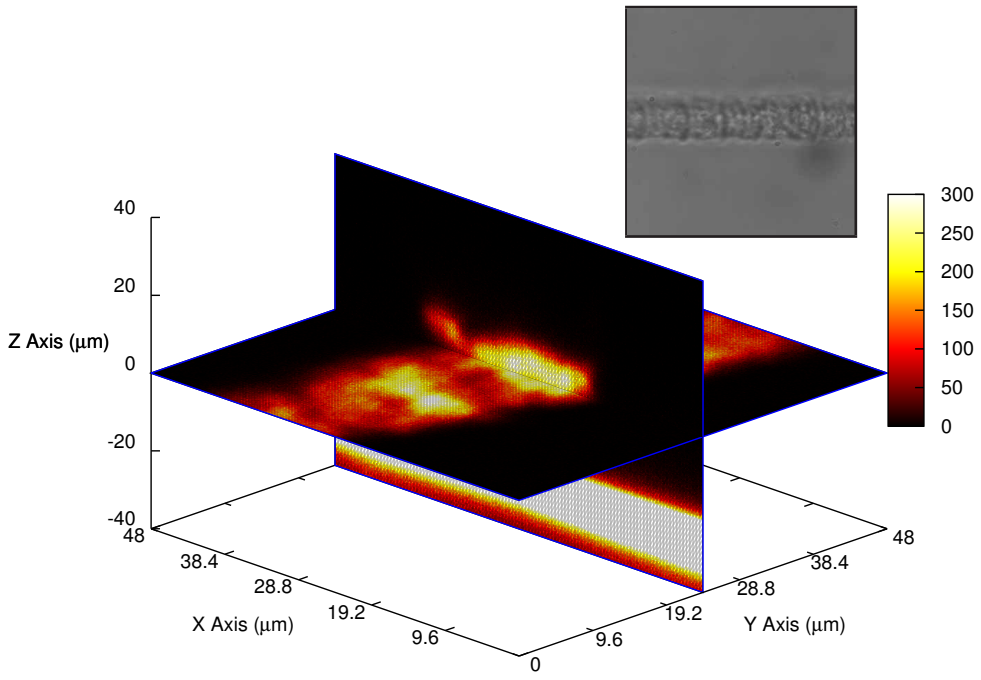

Fig. 3. Combined *x-y* and *x-z* THG scan of features created through spatio-temporal femtosecond micromachining in a fused quartz slide. THG images are limited to 300 photon counts, while the white light image is shown in upper right.

extreme of the axial scan. Notably, the resolution in the *x-z* scan plane image is determined by this axial resolution of the system, while the *x-y* image is determined by the lateral focus spot size.

It is worth noting that the *x-z* scan greatly simplifies locating optimal planes for *x-y* imaging. Without *x-z* imaging it is generally necessary to step the axial position of the stage while taking scans in order to find the planes where the features of interest are most prominent. This time consuming process can be avoided with a simple *x-z* scan, which readily shows the depths that correspond to the features of interest. The capability to perform imaging in this manner also easily generalizes to arbitrary plane scanning, permitting unique explorations within scattering specimens.

3. Four beam differential laser scanning microscope system with remote focusing

3.1. Experimental setup

By utilizing remote focusing, we can effectively extend the imaging capability to four focal planes by coupling the output of our multi-beam Yb:KGW laser [16] to the optical system shown in Fig. 4. This oscillator design adds significant capability and scalability to our multifocal approach. The folded design minimizes the laboratory footprint, and the six beam output eliminates the need for an external optical multiplexer. The beams are extremely energetic (up to 11 nJ per pulse per beam), enabling deep penetration into highly scattering specimens. By virtue of the oscillator geometry, the pulses of these beams are all delayed by

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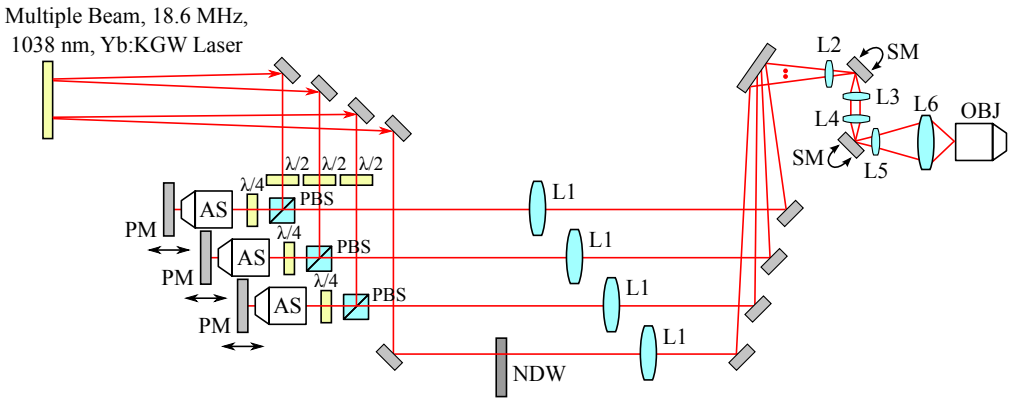

Fig. 4. Four beam remote focusing microscope layout. $\lambda/2$: Half Wave-plate, PBS: Polarizing Beam-splitter, $\lambda/4$: Quarter Wave-plate, AS: Asphere, PM: Piezoelectric Mirror, L1: 400 mm Lens, L2: 100 mm Lens, L3: 40 mm Lens, L4: 40 mm Lens, L5: 35 mm Lens, L6: 200 mm Lens, NDW: Neutral Density Wheel, SM: Scan Mirror, OBJ: Objective

6 ns relative to one another. Such large delays are a result of the extended cavity design. In contrast, standard oscillators produce only a single beam with energies on the order of 10-20 nJ, and the pulses are spaced by 10 to 12 ns. Thus, splitting a beam from a standard oscillator to create six individual beams would decrease the inter-pulse spacing to 1.6-2 ns and, as a result of losses in the external beam-splitter array, the per beam energy would also decrease. Optimistically assuming 90% throughput of the beam splitter / delay line array, such a system would result in 1.5 to 3 nJ per beam. Notably, a tight inter-pulse spacing would negate the use of most fluorophores, as the fluorescence lifetimes would exceed the inter-pulse spacing and make single-element detection impossible. Therefore, a standard oscillator design limits the practical scalability of our multifocal approach and necessitates a custom-built oscillator.

As shown on the left half of the system schematic (Fig. 4), we have implemented remote focusing on three of four excitation beams. The four beams are then angularly multiplexed to the back of the excitation objective using the optical system described by Sheetz et al [16]. This technique is commonly employed for beam combining in multi-focal microscopy [2–14, 16].

Each of the three adjustable beams uses a 0.65 NA asphere (New Focus 5721-H-B) focused onto a zero-degree, high reflector (CVI Melles Griot TLM-1053-0-0643) that is attached to a piezoelectric actuator (Thorlabs PAS100). Each actuator is connected to a separate channel of a three axis open-loop piezo controller (Thorlabs MDT693A), which is adjusted manually. The excitation objective is a 0.65 NA Zeiss A-Plan (40x/0.65).

3.2. Imaging results

Fig. 5 illustrates the acquisition of four two photon excitation fluorescence (TPEF) images all acquired simultaneously using the system shown in Fig. 4. The maize specimen used in these images has been engineered to express the Citrine variant of yellow fluorescent protein (YFP), which is efficiently excited by the 1038 nm wavelength provided by the Yb:KGW laser. The four focal planes are uniformly separated in the axial direction by approximately 7 μm. The angular multiplexing necessitates that each focal plane is offset laterally relative to one another, with the green circle shown in each image serving as a guide for the relative lateral offset between any two images. Each of the individual excitation beams measured 14 mW average power (18.6 MHz repetition rate), as measured after the excitation objective, for all the images acquired in this and the following image series. This beam power and pixel dwell time was

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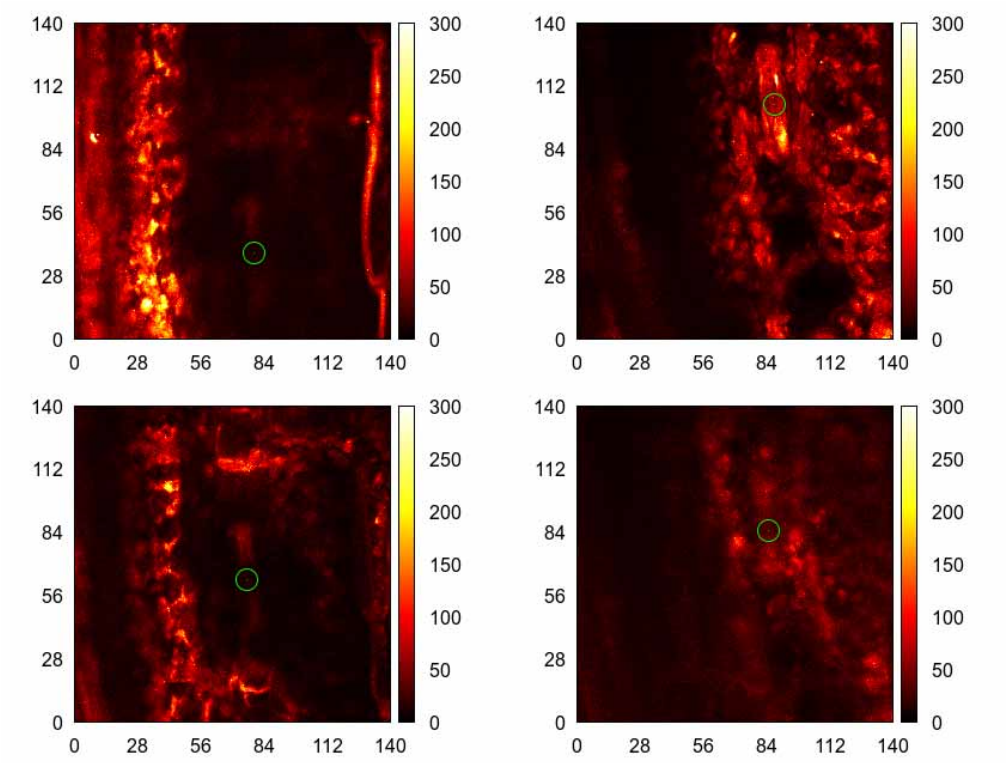

Fig. 5. Single-scan excerpt from a video illustrating simultaneous acquisition of four focal planes, the x and y axes are in microns and the intensity map is in photon counts. This video (Media 1) demonstrates how the focal planes can be programmatically adjusted by sweeping one of the focal planes through the range of the other three. A large dynamic range is achieved by using a frame exposure time of 40 seconds, with an average per-beam power of 14 mW in order to avoid damaging the specimen.

adopted in order to be below the damage threshold of the specimen and achieve a reasonable dynamic range in each frame. For this sample, and these exposure conditions, we typically have a background of 2-3 photons per pixel with a maximum signal of up to $\sim$ 800 photons in a pixel. To date we have achieved pixel dwell times with this multifocal imaging system as short as 0.9 μs ($\sim$ 60 frames/s for a 128x128 scan region). An important feature of our imaging system is that the pixel dwell time can be conveniently changed to produce optimized images based on the damage threshold of the specimen and the efficiency of the optical nonlinearity being employed to generate image contrast.

To demonstrate the utility and simplicity of remote focusing, a single focal plane is varied (upper left image) relative to the fixed focal position of the other three planes, Fig. 5 (Media 1). By tracking a key feature in the frame (such as that shown in the green circle) it is clear that the axial position shifts into (and then out of) the same focal plane of each of the other images as the focus is continuously varied.

Fig. 6 (Media 2) is, once again, a four image series that is acquired simultaneously with the focal planes uniformly offset by approximately 7 μm. In this series the offset is not varied, but rather a time series is taken revealing dynamic behavior at four different levels. The time steps in this series is ~40 seconds, an example of four-dimensional imaging.

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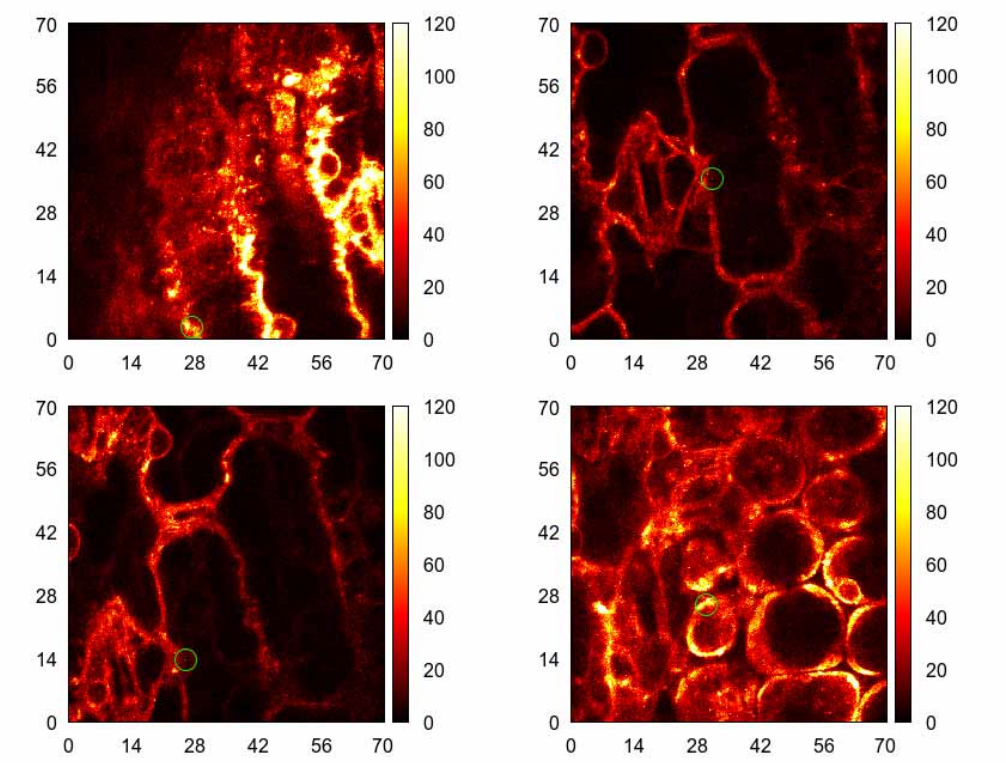

Fig. 6. Single-scan excerpt from a movie of a living corn maize specimen demonstrating the simultaneous acquisition of four focal planes, the x and y axes are in microns and the intensity map is in photon counts. In this video (Media 2) the focal planes are adjusted to a static $\sim 7\,\mu$m offset and a live sample is imaged over time. Each frame is captured with a 40 second exposure time, for a 14 mW per-beam average power, in order to produce significant image contrast without harming the sample.

A limitation of the four beam system is the programmable depth. In this case we have coupled the remote focusing system to a pre-existing optical system which optically multiplexes the beams together at the back of the excitation objective. As a result we had to employ imaging systems with non-unity magnification, which results in limiting the maximum excursions that we can achieve to approximately 20 micrometers. In order to achieve the full excursion depth (as demonstrated in our dual focus system, Section 2) it will be necessary to redesign the optical multiplexer in conjunction with the remote focusing system to ensure 1:1 image conditions throughout, thereby maximizing our axial scan depth.

4. Conclusion

Differential microscopy facilitates volumetric visualization by mitigating scattering ambiguities common to traditional multifocal multiphoton imaging systems. The marriage of differential microscopy with remote focusing, as demonstrated in this work, extends the utility of both of these technologies by enabling three-dimensional volumes to be explored with programmable control of the lateral and axial scan directions. For example, by synchronizing the remote focusing direction with the x-scan direction we have demonstrated the acquisition of image planes orthogonal to the traditional lateral scan plane. To date we have scaled remote focusing

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to a four beam system, enabling the simultaneous capture of entire image volumes as a function of time – the first realization of fully programmable 4-D multiphoton imaging. It is also possible to further generalize these conditions, such as by adding focal planes or by rotating the scan planes to selectively target interesting features. Consequently, this technology holds significant promise as being an important new imaging tool for the efficient exploration of biological phenomena.

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