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THREE-DIMENSIONAL RANDOM ACCESS MULTIPHOTON MICROSCOPY FOR FAST FUNCTIONAL IMAGING OF NEURONAL ACTIVITY

Gaddum Duemani Reddy^{2,1}, Keith Kelleher³, Rudy Fink¹, and Peter Saggau^{1,2}

¹Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030

²Department of Bioengineering, Rice University, Houston, TX 77005

³Department of Biology and Biochemistry, University of Houston, TX 77004

Abstract

The dynamic ability of neuronal dendrites to shape and integrate synaptic responses is the hallmark of information processing in the brain. Effectively studying this phenomenon requires concurrent measurements at multiple sites on live neurons. Significant progress has been made by optical imaging systems which combine confocal and multiphoton microscopy with inertia-free laser scanning. However, all systems developed to date restrict fast imaging to two dimensions. This severely limits the extent to which neurons can be studied, since they represent complex three-dimensional (3D) structures. Here we present a novel imaging system that utilizes a unique arrangement of acousto-optic deflectors to steer a focused ultra-fast laser beam to arbitrary locations in 3D space without moving the objective lens. As we demonstrate, this highly versatile random-access multiphoton microscope supports functional imaging of complex 3D cellular structures such as neuronal dendrites or neural populations at acquisition rates on the order of tens of kilohertz.

INTRODUCTION

Developing a thorough understanding of the computational capabilities of a single neuron is one of the greatest goals in the field of experimental neuroscience. However, any attempt to study neuronal physiology with subcellular resolution is complicated by four fundamental technical challenges. First, the small size of many neuronal processes that are highly important for neuronal computation prohibit direct electrical recording. For hippocampal CA1 pyramidal neurons in particular, an estimated 50% of all synapses, including approximately 80% of the Schaffer collateral synapses, are located on thin dendrites that are less than one micron in size¹ and thus inaccessible to patch pipettes. Second, in brain slices or intact cortex, neurons of interest can be located several hundreds of microns deep inside the tissue, requiring any recording method to be capable of isolating them from their environment. Third, relevant neuronal signals, such as transients of membrane potential and ionic membrane currents, happen on the order of milliseconds, necessitating recording methods with high temporal resolution. Fourth and finally, the space occupied by these cells can span hundreds of microns in all three dimensions², further restricting optimally effective recording methods to only those capable of monitoring from a volume. These four challenges have stimulated the development of increasingly advanced fluorescence imaging techniques, which have become commonly valued tools in the arsenal of the experimental neuroscientist.

Correspondence should be addressed to PS (psaggau@bcm.edu).

Indeed, several studies have met the first two challenges listed above by using the diffraction-limited spatial resolution and optical sectioning capabilities provided by confocal and multiphoton microscopy to study physiological dynamics in thin oblique dendrites³⁻⁶, as well as dendritic spines⁷⁻¹³. Recent studies have further extended the temporal resolution of these techniques by replacing galvanometer-driven beam steering mirrors with inertia-free acousto-optic deflectors (AODs)¹⁴⁻¹⁸. Such AOD-based scanning has been previously used to study neuronal physiology in cell cultures^{19,20}, and has also been implemented for ultraviolet (UV) photolysis of caged compounds in cultures²¹⁻²³. The combination of multiphoton/confocal microscopy with this form of beam scanning^{15,16} represents the only approach that truly meets the first three challenges listed above. This is because the random access capabilities and high temporal resolution provided by AOD-based scanning allows for effectively simultaneous imaging of several sites on a neuron²⁴, whereas similarly equipped galvanometer-based scanning methods are limited to nearby locations and line-scans.

However, to date, no technique has effectively met all four challenges, primarily because it was only possible to image from a single focal plane with sufficient temporal resolution to track neuronal signals. Indeed, even for AOD-based scanning techniques, the temporal resolution enhancement and random access capabilities applied only to a single focal plane. In such techniques, scanning different axial planes requires the objective lens to be physically moved with an actuator, such as a stepper motor or a nanopositioner, a process which is temporally limited by the combined inertia of objective lens and actuator. Several methods have been developed to quickly change the focal position, and thus allow for axial scanning, including deformable mirrors²⁵, variable focus liquid-filled lenses²⁶, and simultaneous spatial and temporal focusing^{27,28}. However, none have the ability to simultaneously scan in the lateral dimension, and none have been demonstrated to functionally image parameters of neuronal activity. In addition, very few methods have achieved acquisition rates faster than one kilohertz. Other techniques which extend the depth of field to provide a maximum projection image of the neuron, such as axicon-based scanning^{29,30}, prevent the functional imaging of structures which overlap axially and drastically increase the power requirements for multiphoton imaging. In addition, a recent technique of strategically combining axial movement of the objective lens via a piezoelectric actuator with lateral scanning using galvanometers has been shown to be effective in imaging somatic calcium transient *in vivo*³¹. However, although this technique employs sinusoidal axial movements, it is still constrained by the inertia of the objective lens, which limits the acquisition rate of the system.

Given the extent of these various constraints in imaging highly complex structures such as neuronal dendrites, we recently developed a strategy that utilizes a unique arrangement of multiple AODs to allow for inertia-free random-access scanning in three dimensions (3D)³². In this proof-of-principle study, we demonstrated that this technique allows for scanning acquisition rates on the order of tens of kilohertz for user-selected sites of interest that occupy many different positions within a volume. In addition, we showed how this scheme had the inherent benefit of compensating for spatial dispersion, an effect degrading the spatial resolution of AOD-based multiphoton microscopy that usually requires an external source for compensation^{33,34}. Another recent report has utilized a simpler version of this approach for fast scanning of neurons in 3D. However, this reduced method does not allow for true random-access imaging in all three dimensions, and therefore has not demonstrated fast 3D functional imaging³⁵.

In the present article, we implement our 3D scanning technique³²⁻³⁴ and combine it with multiphoton microscopy to create the first system capable of overcoming all four limitations listed above and demonstrate the first evidence of random-access functional imaging of neurons in a volume of tissue. Furthermore, we use this system to concurrently monitor calcium

transients at several different lateral and axial locations on individual pyramidal neurons in brain slices.

RESULTS

3D Acousto-Optic Scanning Principle

To generate random access scans in three dimensions, we utilized a system of AODs with chirped (i.e., continuously changing in frequency) and counter-propagating acoustic waves. This approach is different from the method used for two-dimensional (2D) random access applications^{14-17,19-21}, in which a fixed frequency is supplied to a single AOD per scan dimension (Figure 1A), which results in a deterministic deflection angle θ , as specified by Equation 1,

$$\theta = \frac{\lambda f}{v}, \quad \text{Eqn. 1}$$

where λ is the wavelength of the incident light and f and v are the frequencies and velocities of the propagating acoustic waves, respectively^{36,37}. As detailed in an earlier work³², when two AODs are utilized per lateral dimension and the acoustic waves are counter-propagating and chirped, as shown in Figure 1B, two independent effects are observed. First, there is a deterministic lateral deflection of the laser beam with an angle specified by Equation 2,

$$\theta = \frac{\lambda(f_{1c} - f_{2c})}{v}, \quad \text{Eqn. 2}$$

where f_{1c} and f_{2c} are the center acoustic frequencies in the first and second deflectors respectively. Second, a beam convergence or divergence is introduced, since an effective cylindrical lens is generated with a focal length, F_{AOL} , as described by Equation 3,

$$F_{AOL} = \frac{v^2}{2\lambda\alpha}, \quad \text{Eqn. 3}$$

where α , expressed in Mhz/ μ s, is the chirp parameter, or the rate of change in frequency^{32,38}. By utilizing two orthogonal pairs of deflectors, for a total of four AODs, we created a variable focal length spherical lens, which can be used to slightly change the collimation of a beam entering the objective lens, thus changing the axial focal positions. Since the deflection angle, θ , depends only on the difference between the center acoustic frequencies in each pair of AODs while the focal length of the acousto-optic based lens, F_{AOL} , depends only on the rate of change in the acoustic frequencies, this approach allows for independent axial and lateral focal positioning. This feature, when combined with multiphoton excitation principles, results in the ability to precisely measure fluorescence from anywhere within a volume.

3D Random Access Multiphoton Microscope System Design

Optical Layout—Using the principle of 3D AOD-based scanning described above, we constructed a multiphoton microscope system as shown in Figure 2A. The system is built around the framework of an upright epifluorescence microscope (*E600FN*, Nikon), similar to previous systems we have developed¹⁵. It contains a laser scanner based on four large aperture AODs for positioning in both lateral dimensions, and to create a variable focal length spherical lens for positioning in the axial dimension. The laser source is provided by a Titanium:Sapphire (Ti:S) laser (*Mira HP*, Coherent) pumped by a 532nm solid state CW laser (*Verdi 18*, Coherent)

to generate ultra-fast pulses around 800nm with a mode-locked average power of approximately 4W. A small portion of the beam (<1%) is sent to a spectrum analyzer (*E201LSA03*, Imaging and Sensing Technologies) for bandwidth analysis ($\Delta\lambda \sim 10\text{nm}$). In addition, an autocorrelator (*Mini*, APE) is used to measure the pulsewidth ($\sim 200\text{fs}$) and is removed from the beam path during experiments. The $\sim 3\text{mm}$ beam is magnified 3-fold and directed through a series of four AODs, four 1:1 telescopes, and a 1.2:1 scan angle magnifier, before reaching the back focal aperture (BFA) of a 60X (NA 1.0) water immersion objective lens (*CFI Fluor*, Nikon). The telescopes and deflectors are arranged such that each AOD is located one focal distance away from the lens of a telescope, thereby creating a series of 4-f systems. This layout effectively images each deflector onto the next, thereby eliminating the distance between deflectors oriented in the same direction (i.e. the X deflector pair or the Y deflector pair), which would otherwise reduce the quality of the AOD-based variable focal length lens. In addition, this optical layout images the output of all four deflectors onto the BFA of the objective lens, to establish a pivot point for lateral displacement, and a beam waist location for axial displacement of the laser beam. The scan angle magnification serves to increase the effective lateral scan field to $\sim 200\mu\text{m} \times 200\mu\text{m}$ and also de-magnifies the 9mm beam diameter to closer match the 7.5mm BFA. The mirror system located between the second and third deflector, rotates the beam by 180 degrees and thereby results in counter-propagation of the acoustic waves in corresponding AOD pairs. The AOD-based scanner was coupled to the upright microscope through a modified camera port located on top of the scope ("*Double Port*", Nikon). Within this unit, excitation light was reflected by a short-pass dichroic mirror (*750DCSPXR*, Chroma), which also allowed for visualization of the specimen by infrared-sensitive camera (*IR-1000*, DAGE-MTI) and infrared light transillumination (not shown in Figure 2A). This visualization scheme was useful for patching neurons before imaging experiments were performed. Excitation light was subsequently transmitted through a long-pass dichroic (*700DCXR*, Chroma) mirror located in the filter slider of the epifluorescence unit ("*Intermediate Port*", Nikon) before reaching the BFA of the objective lens. Emission light was reflected by this long-pass dichroic mirror and transmitted through emission filters (600/200nm or a 500/100nm + 535/50nm combination, Chroma) to the epi-illumination port. There it was focused onto the photocathode of a photomultiplier tube (PMT) module (*H9307-3*, Hamamatsu) using a 5.6X demagnification telescope, which fitted the image of the 7.5mm objective lens BFA onto the 3.7mm $\times$ 13mm PMT photocathode.

Acousto-Optic Deflectors—We used large optical aperture (9mm $\times$ 9mm) slow shear acoustic wave TeO_2 AODs (*OAD-1121*, Isomet). This material was chosen because of its high acousto-optic figure of merit, which allows for large apertures devices with high interaction bandwidth ($\sim 40\text{Mhz}$) and a high diffraction efficiency ($\sim 70\%$ into the first order). The large size of the apertures in turn increases the resolution of the AODs, as determined by the number of resolvable points (NRP), by reducing the diffraction-limited spot size³⁷. One consequence of using this acousto-optic material, however, is that each deflector rotates the polarization of the beam by 90 degrees³⁶. This requires that the AODs be arranged in a specific order to match their incident polarization, with orthogonally oriented deflectors placed in sequence. The polarization for the first deflector was adjusted using a half wave plate (*AHWP05M-950*, Thorlabs).

Each AOD is driven by a radio frequency (RF) signal generated by a voltage-controlled oscillator (*VCO-200A*, Isomet). These particular VCOs have high tuning linearity ($<0.1\%$ deviation over a 50Mhz range) and very high tuning slew rate ($>10\text{Mhz}/\mu\text{s}$), which exceeds the maximum chirp parameter needed for our AOD-based focusing (defined as the entire frequency range of 40Mhz over the aperture time of 10 μs , or 4Mhz/ μs). In order to reach the RF saturation power of approximately 2W for the deflectors, the output from the VCOs is amplified (*IA-100-3* + *DA-104-2*, Isomet).

Scanning Control and Data Acquisition—The control voltages to the VCOs were supplied by the four analog output channels of a multifunction data acquisition card (NI-6259, National Instruments). The high analog output rate (1.25 MHz) resulted in relatively smooth frequency ramps being generated by the VCOs. The fluorescence signal is detected, current-to-voltage converted, and amplified by the PMT module and custom designed electronics, to be received by an analog input line from the data acquisition card. The process of beam scanning and data acquisition is controlled by custom software designed in the Dot Net 2005 platform (Microsoft). The software synchronizes the hardware tasks and allows the user to perform a 3D raster scan to locate regions of interest and subsequently select recording sites within those regions.

Axial Position Scan

We determined the extent to which measured axial position deviations agreed with theoretical values based upon Gaussian optics analysis. For this purpose, we used the scanner to image a layer of 200nm fluorescent beads (*T-14792*, Molecular Probes) which we selectively positioned at different axial distances from the objective lens. Practically, this was done by acquiring image stacks of the bead layer using an objective lens stepper motor (*Remote Focus Accessory*, Nikon) around axial locations determined by the AOD scanner. The initial stack was imaged at the inherent focal position of the objective lens, by using unchirped acoustic waves in each AOD. For this initial stack, the microscope was adjusted manually to bring the bead layer into focus. Subsequently, the axial location of the specified section being imaged was moved by changing the chirp parameter in each AOD as described above. This changed the collimation of the beam entering the BFA of the objective lens and shifted the axial location of the focus relative to its inherent focus. The objective lens was then repositioned to bring the bead layer back into focus using the stepper motor, and another stack was acquired. The axial offset of the objective needed to refocus the bead layer, as well as the position of maximal fluorescence in the stack, were used to determine to what extent the axial focus position had shifted. As shown in Figure 2B, excluding the inherent focus of the objective, eight separate axial positions were measured using this method across a 50 μ m range. As can be seen from Figure 2B, the measured positions agreed very closely with the theoretical values. The theoretical predictions were based on an ABCD matrix theory model for Gaussian beam propagation³⁹ which simplified the entire optical layout to a spherical lens with a focal length determined by Equation 3 above, a 1:1.2 scan angle magnifier, and an objective lens.

3D Imaging of Test Preparations

To demonstrate the ability of the microscope system to acquire full 3D images without moving the objective lens, we used two types of fluorescent test preparations, i.e., 10 μ m beads (*F-8836*, Molecular Probes) and ~35 μ m pollen grains (*30-4264*, Carolina Biological Supply Co). Initial tests were done with beads to compare the structural images attained with two separate methods. To obtain a control measurement, we used the AODs only for lateral positioning and the stepper motor for axial positioning. This 3D scanning method, which we define here as “2+1D” scanning, resembles previously used schemes for AOD-based multi-photon laser scanning microscopy^{14,15,17}. The second 3D scanning method, which we define as “3+0D” scanning, used only the AODs for both axial and lateral positioning. For the beads, 100 optical sections were acquired with each method over an axial range of approximately 25 μ m and with a lateral resolution of 250 $\times$ 250 points. Using the image stacks, the beads were 3D reconstructed (AMIRA, Template Graphics Software), and axial as well as lateral projections were created. Typical results are shown in Figure 3A, indicating only minimal differences between the 2+1D and 3+0D scanning methods. To further extend this comparison to larger structures, we performed a similar test using pollen grains. Here we acquired 100 sections over a range of approximately 40 μ m, and reconstructed the pollen grains (*Voltex*, AMIRA). The axial view of the reconstruction and every tenth optical section is shown in

Figure 3B for both acquisition methods. As can be seen from this figure, the image quality produced by the different scanning methods appear similar. More quantitative assessment of the resolution changes can be achieved through comparing the PSFs at different axial locations versus at the objective focus (supplementary Figure 1).

3D Imaging of Neurons

Structural Imaging—Before a meaningful random access scan can be performed, a structural image must be generated from which points of interest can be selected. It would be convenient to generate this image using the $3+0D$ scanning method as well, since this would allow for exact registration between the structural and functional images. Our results from the test preparations suggest that $3+0D$ scanning would give images with relatively the same quality as the $2+1D$ method. To determine the degree to which this holds true for fluorescently labeled cells, we compared structural images of hippocampal CA1 pyramidal neurons obtained with $3+0D$ and $2+1D$ scanning. Figure 4A represents the composite maximum projection image of three separate, overlapping image stacks of a CA1 pyramidal neuron acquired with $3+0D$ scanning at a resolution of $200 \times 200 \times 20$ points and over an axial range of $50 \mu\text{m}$. As can be seen from this figure, thin oblique dendrites are clearly visible. Similarly, Figure 4B shows reconstructed images from a single stack of a CA1 pyramidal cell acquired with $2+1D$ and $3+0D$ scanning (both at a resolution of $200 \times 200 \times 50$ points and over an axial range of $50 \mu\text{m}$), demonstrating similar quality of the structural images produced by the different scanning methods.

To allow for comparisons on a more detailed level, we then zoomed-in on a segment of a thin dendritic branch by restricting the acoustic frequency ranges applied to the deflectors (Figure 4C). We imaged this segment at three different AOD-based focal positions to compare the image quality when the focal plane is adjusted in this manner. First, the beam entering the BFA remained collimated to acquire an image of the segment at the inherent focal plane of the objective lens (Figure 4C middle). Note that this represents the image quality that would be obtained from a single optical section with the $2+1D$ method. Subsequently the collimation was changed using the AODs to position the effective focus approximately $25 \mu\text{m}$ above the inherent focal plane and the objective lens was moved down by the same amount with the stepper motor to bring the dendritic segment back into focus (Figure 4C top). Finally, the collimation was changed to position the effective focus $25 \mu\text{m}$ below the inherent focal plane and the objective lens was accordingly moved up to compensate (Figure 4C bottom). Comparing the images in Figure 4C reveals that small processes, such as dendritic spines, remain visible at all axial locations, despite a marginal reduction in image quality. These results confirm that exact registration during imaging can be achieved by using $3+0D$ scanning to generate the initial structural image of the cell, upon which subsequent functional imaging sites can be selected.

Functional Imaging—To test the ability of the 3D random-access multiphoton microscope to provide multi-site measurements of neural activity, we monitored localized calcium transients in response to back-propagating action potentials (bAPs). An identified CA1 pyramidal neuron was filled by patch pipette with both a fluorescent label and a calcium indicator. Then, a $3+0D$ structural scan was performed over an axial range of approximately $25 \mu\text{m}$. The maximum projection image of this structural scan (resolution $200 \times 200 \times 20$ points), along with specified individual axial sections is shown in Figure 5A. From the acquired stack of structural images, different points along the main apical dendrite were selected for functional imaging as shown in the figure. The functional scan was subsequently performed by visiting only these selected sites with a $15 \mu\text{s}$ positioning time and a $5 \mu\text{s}$ dwell time. The effective acquisition rate is the inverse of the sum of the positioning time and the dwell time, which was $20 \mu\text{s}$ for all our experiments, times the number of points. Since five sites were selected in this

situation, this resulted in effective acquisition rates of approximately 10kHz. Note that, unlike previous 2D AOD-based scanning microscopes, the points selected for the functional scan were from different lateral and axial locations. Indeed, as can be seen from the color-coded axial positions of the selected sites, the axial range of the functional scan sites on this neuron is approximately 10 μ m. Local dendritic calcium influx during a train of three bAPs was monitored by sampling the change in normalized fluorescence signal of the Ca²⁺ indicator OGB-1 at the user-selected sites (Figure 5B).

To demonstrate the random-access performance of the 3D AOD scanner over a larger axial range, as well its ability to image finer oblique dendrites, more functional scans were performed. CA1 pyramidal neurons were structurally imaged as described above over an axial range of up to 50 μ m. As before, the structural scans served as the framework on which specific points were selected throughout the dendrites for functional imaging. In Figure 6A, the full axial range of the functional scan for this particular cell extends to ~30 μ m and image points are taken from both above and below the inherent focal plane of the objective lens. The calcium transients at the different locations in response to the bAP train are displayed next to the identified sites. The acquisition rate was approximately 3kHz. For Figure 6B, a zoomed-in portion of the apical dendrite, consisting of the main apical branch and nearby oblique branches, was scanned at an acquisition rate of about 5kHz. As can be seen from the reconstruction, user selected points can be distinguished even if they are primarily only axially displaced from each other, which would be not possible in 3D scanning methods that drastically extend the depth of field^{29,30}. Figure 6C shows an even more zoomed-in image of a branching oblique dendrite. In this case, sites were selected for functional imaging along both the dendrite and a dendritic spine. The acquisition rate was ~3kHz.

DISCUSSION

We have developed a random access multiphoton microscope that allows for fast 3D imaging without physically moving scan mirrors or the objective lens. This is achieved by a unique arrangement of four AODs for laser beam steering. We have used this microscope for structural and functional imaging of pyramidal cells. We have recorded calcium transients from different lateral and axial positions on a neuron with acquisition rates that are in the kilohertz range by utilizing the random access capabilities of this inertia-free 3D laser scanner.

One current limitation of our approach is the restriction to a 50 μ m axial scan range when using a high magnification objective lens. While this exact number is flexible and the axial range can be stretched further, we have noticed a substantial drop in excitation power at further axial distances. This is primarily due to the birefringent diffraction principle of the utilized slow shear TeO₂ AODs. A hallmark of this diffractive effect is a significantly narrow incident angular aperture. This constraint reduces the diffraction efficiency of uncollimated incident laser beams, such as the beams at the third and fourth AODs of our scanner when the axial position moves away from the inherent objective focus. By the acoustic bandwidth considerations alone, an axial scan range of approximately 200 μ m and an lateral scan range of approximately 350 μ m should be feasible³². One possibility to avoid this effect would be to use deflectors whose diffraction is not birefringent-based, unfortunately, with larger angular apertures, such AODs have significantly lower overall diffraction efficiencies. One intriguing possibility would be to utilize specialized wide-angular-aperture deflectors. These deflectors have been shown to provide high diffraction efficiencies while maintaining larger angular apertures^{40,41}, thus making them optimal for AOD-based focusing applications. We are currently working on an improved scanning scheme with extended axial scan range, utilizing custom-made wide-angular-aperture AODs.

In general, larger axial ranges would also reduce the effective spatial resolution. Indeed, in addition to minor reductions in effective objective numerical aperture³², there is also an anticipated increase in spherical aberration as the excitation beam at the BFA of an infinity-corrected objective lens deviates from the perfectly collimated condition⁴². The amount of spherical aberration introduced in this work did not extensively effect the imaging resolution over the used axial range, as shown in supplementary Figure 1, with the largest measured change in lateral resolution being a factor of 1.76 and the largest change in axial resolution a factor of 1.52. While such resolution changes had a small but noticeable effect on structural imaging, as can be seen in Figures 3 and 4, the effect on functional imaging was negligible, since the tiniest functional units we are interested in, i.e., spines, are not significantly smaller than our largest spot size. Other groups have shown minimal reductions in spherical aberration from collimation changes in water immersion lenses over a similar axial range⁴³. With larger axial ranges, however, it is generally anticipated that spherical aberration will become more important. In such cases, it might prove useful to utilize a reverse Gaussian aperture⁴⁴ to preserve the resolution over the entire axial range at the expense of a mildly reduced resolution at the inherent focal plane.

One issue we did not address in this context is temporal pulse broadening. While the scanning mechanism we employed inherently compensates for spatial dispersion³², it does not compensate for temporal dispersion. Consequentially, the pulsewidth is broadened from 200fs when exiting the Ti:S laser to approximately 1.8ps at the BFA. This required an increase in laser power for two-photon fluorescence excitation, and average powers of approximately 40-100mW at the BFA were used for these studies. This power increase also compensated for the decrease in intensity at positions not at the focal point of the objective due to increased spot sizes (see supplementary Figure 1), which would otherwise also reduce excitation efficiency. Notably, this amount of power was approximately 10%-25% of the amount available at the BFA, thus we were not power limited by the broadened pulsewidth. In addition, while it might seem that the required higher power levels would lead to increased photodamage, previous studies have shown equivalent levels of photodamage in multiphoton microscopy when using pulsewidths ranging from less than hundred femtoseconds to several picoseconds⁴⁵. It has even been suggested that potential photodamage can be reduced with longer pulsewidths⁴⁶. For these reasons, combined with the physical impracticality of a prism-based prechirper large enough to compensate for the $\sim 80,000\text{fs}^2$ group velocity dispersion (GVD) introduced by the four AODs, an external temporal compensation device was not utilized. On the other hand, it has been also been suggested that using narrow pulses in multiphoton microscopy will lead to increased penetration depth⁴⁷, since higher peak powers can be applied to deeper tissue. Thus, with increasing axial ranges, or with “in vivo” imaging where more tissue is transversed⁴⁸, external temporal dispersion compensation might become increasingly valuable, in which case, compensation scheme utilizing diffraction gratings⁴⁹ or photonic crystal fibers⁵⁰ offer possible methods for generating the large amount of compensatory negative GVD values. Another possibility is to use moderately longer pulselengths than what we used in this experiment to start with, which minimizes both the temporal dispersion and spatial dispersion induced by the system. However, these optimal mid-range femtosecond pulse lengths are not widely commercially available on most laser systems and typically require either off-label manipulations to the laser cavity¹⁵ or custom designed laser systems³⁵.

Two recent manuscripts have reported the development of other methods for 3D functional neuronal recordings, both of which are significantly different from the method proposed here. The first is from Gobel et al., who combine a scanning system consisting of galvanometer driven pivoting mirrors for lateral imaging and a piezoelectric actuator for axial imaging with a novel scanning strategy³¹. In essence, this technique represents the furthest extent to which inertia-based scanning techniques can be taken. Indeed, when used for scanning neuronal populations, it has been shown that up to 90% of user-selected cells can be imaged at acquisition

rates on the order of 10Hz. The remaining fundamental limitation is the dependence upon actuator-and galvanometer-based scanning technologies. In fact, even the fastest piezoelectric actuators are only capable of speeds around 100Hz, if not slowed down by the inertia of a microscope objective. However, at the higher speeds, the vibration from physical movement of the objective lens can easily disturb image quality and disrupt biological samples. In contrast, the AOD-based scanning approach presented here is easily capable of acquisition rates that are in the kilohertz range, without imparting any physical vibrations to the sample or the image. While the present 50 μ m range of our scanner is smaller than the axial range of many piezoactuators, which can be 100s of microns, as mentioned above, this axial range can be extended to similar values by using either, a lower magnification objective lens, wide-angular-aperture deflectors, or a combination of both.

The second manuscript which demonstrates a method only for fast 3D scanning is by Vucinic et al, in which two AODs are used to quickly scan a volume³⁵. This approach can be seen as a simplified version of the scanning scheme presented here, wherein the acoustic frequencies applied to the first X and Y deflectors are kept constant, or the first two deflectors are bypassed altogether, but chirped frequencies are applied to the latter X and Y deflectors. Since these acoustic frequencies are chirped, the laser beam after the AODs is no longer collimated, as shown in Figure 1b. In addition, similar to the method presented here, the degree of collimation, and hence the axial position can be changed by changing the degree of chirp. However, unlike the method presented here, there is no secondary set of deflectors in the path. As a result, there is a continuous lateral movement of the laser beam that is not compensated and forces the scanner into a continuous “raster” mode. While this approach does allow for 3D structural imaging, it has two consequences for functional imaging. First, it limits the effective speed of the system since time is wasted at sites that are not of interest as this is true with all raster scanning systems. Second, this scheme drastically reduces the dwell time per point of interest. This severely limits the number of detected photons, and reduces the achievable signal-to-noise ratio, a fact which the authors acknowledge. These two consequences make this approach non-optimal for functional 3D imaging. Indeed, while Vucinic et al have shown functional imaging signals, they have been able to do this only when not scanning in three dimensions. Thus, when acquiring functional data, their imaging scheme resembles previously published 2D schemes¹⁵. In this regard, while both approaches allow for fast 3D structural imaging, unlike our approach, their approach does not permit fast 3D functional imaging, where high scanning speeds are arguably more important.

In summary, we have developed a multiphoton microscope that represents the first device we know of capable of recording from multiple sites in a tissue volume at acquisition rates that are in the tens of kilohertz range. We have utilized this instrument to monitor calcium dynamics in a single neuron. Such experiments have previously be limited to a single focal plane, which often restricts researchers to very few sites as dendritic processes can rapidly pass through this plane. Our approach can also be combined with similarly designed 3D random-access photolysis of caged compounds, which opens the door for countless experiments examining the spatio-temporal dynamics of neural computation. Additionally, this technique can be immediately applied to a variety of other neurophysiological situations, most notably in the study of cells populations. In this case lower magnification would be employed, and indeed, we have previously shown an axial range of approximately 1mm when the AOD-based system is combined with a 10X objective, giving a large axial range over which cells could be imaged. Given its applicability to a wide range of neurophysiological experiments, this novel imaging tool represents an important technological advancement in the current experimental arsenal of neurophysiologists.

METHODS

Brain slice preparation

Hippocampal brain slices were obtained from 4-6 wk old Sprague Dawley rats and prepared in accordance with the guidelines of the National Institutes of Health as approved by the animal care and use committee of Baylor College of Medicine. Rats were anesthetized with a mixture of ketamine, xylazine, and acepromazine, and perfused trans-cardially with a 2-4°C solution consisting of (in mM) 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 7mM dextrose, 100 choline chloride, 1.3 ascorbate and 3 pyruvate. The brain was dissected, hemisected and mounted in the holding chamber of a tissue slicer (Vibratome 100, Ted Pella) in this same solution. Hippocampal brain slices of 350µm thickness were cut and subsequently transferred to a holding chamber containing an artificial cerebrospinal fluid solution containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 2 MgCl₂, and 10 dextrose at 35°C. The solution was bubbled with a 95% O₂-5%CO₂ mixture. Slices were held at this temperature for 45 minutes before moving them to a room temperature recording chamber 20 min before use. For experiments, the solution was held at 30-34°C using an in-line temperature controller (TC-324B, Warner Instruments).

Electrical and Optical Measurements

Patch pipettes were pulled using a pipette puller (*Model P-97*, Sutter Instruments) to tips with resistance values between 3-5MΩs. This was confirmed by measuring voltage values using the seal test of the patch clamp amplifier (*Axo-Patch 200A*, Axon Instruments). These pipettes were filled with solution containing (in mM) 120 K-Gluconate, 20 KCl, 10 HEPES, 2 MgCl₂, 4 Mg₂GTP, 0.3 NaGTP and a combination of a structural label, 50µM Alexa 594 and a Ca²⁺ indicator, 200 µM OGB-1 (both Molecular Probes). Pyramidal neurons of interest from the CA1 region of the hippocampus were identified and patched in the whole-cell configuration using widefield IR-DIC imaging at depths approximately 50µm to 100µm below the surface of the slice. This was accomplished by removing the filter slider holding the short-pass dichroic mirror shown in Figure 2, allowing trans-illumination light to reach an IR-sensitive camera mounted on the top of the microscope. Measured changes in Ca²⁺ indicator fluorescence were normalized using ΔF/F values, which were calculated according to Equation 4 below.

$$\frac{\Delta F}{F} = 100\% \times \frac{F - F_{baseline}}{F_{baseline} - F_{background}}, \quad \text{Eqn. 4}$$

where F is the value of PMT-generated voltage signal at a given time point, $F_{baseline}$ is the average value of the pre-stimulus signal, and $F_{background}$ is the average value of the measured dark noise from the PMT. Signals were low-pass filtered to 50Hz using a finite impulse response (FIR) filter to remove residual shot noise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. 2D versus 3D AOD Scanning

(a) 2D scanning: *Left*, angular deflection ($\theta(x,t)$) of a collimated laser beam by a single AOD with a fixed frequency (f) acoustic wave. *Right*, two-point random-access lateral scan of an ultra-fast laser beam focused by an 10X objective lens into a cuvette containing fluorescein solution, resulting in two distinguishable focal spots in a single axial plane (*inset*, zoomed-in view). *Bottom*, fast scanning with 2 orthogonal AODs is restricted to a single plane.

(b) 3D scanning: *Left*, change in collimation (focal length F_{AOL}) and angular deflection ($\theta(x,t)$) by counter-propagating chirped acoustic waves (f_1, f_2) in a pair of AODs. *Right*, two-point random-access axio-lateral scan, resulting in two distinguishable focal spots at different

axial planes (*inset*, zoomed-in view). *Bottom*, 2 orthogonal pairs of AODs support fast scanning within a volume.

Figure 2. Optical Layout and Axial Scan Range

(a) Imaging system containing an ultrafast pulsed laser (Ti:S Mira HP), pulse diagnostic devices (Spectrum Analyzer, Autocorrelator), beam shaping optics (Beam Expander, Scan Magnification), the 3D AOD Scanner (see inset), an upright epifluorescence microscope (Dichroic Mirrors, Objective Lens), and the detection unit (Demagnification, Emission Filter, PMT). *Inset*, 3D AOD Scanner consisting of polarization control ($\lambda/2$ Plate) and 4 AODs with relay telescopes (Scan X1,2, Y1,2 AODs). Not shown, located above the short pass dichroic mirror, an infrared-sensitive camera, used for transillumination visualization of the specimen during patching.

(b) Predicted (solid line) and measured (crosses) axial distance of the focal position from the inherent focal plane of the objective lens.

Figure 3. Structural Imaging with Different 3D Scanning Modes

(a) Axial and lateral projections of a fluorescent bead (10µm diameter) imaged with combined 2D AOD lateral scanning and stepper motor axial positioning ("2+1D" mode), and with 3D AOD scanning ("3+0D" mode).

(b) Axial projections (*left*) and selected optical sections (*right*) of a reconstructed pollen grain (~35µm size) imaged in both the 2+1D and the 3+0D mode (scale bars, 10µm).

Figure 4. Structural Imaging of Neurons

(a) Axial projection of a neuron filled with a fluorescent label (Alexa 594), acquired in 3 separate image stacks using the 3+0D scanning mode (axial range, 50μm; scale bar, 50μm). (b) Full projections of single image stacks from another neuron, acquired in both the 3+0D and the 2+1D mode (XY scale bar, 50μm; ZY scale bar, 10μm). (c) Dendritic segment imaged in 3+0D mode with high resolution (zoom-in) at three different AOD-controlled focal distances. *Middle*, reference image, taken at inherent focal distance of the objective lens (reference plane). Test images, taken at AOD-controlled effective focal planes 25μm above (*top*) and 25μm below (*bottom*) the reference plane with the objective lens moved in the opposite direction to compensate (scale bars, 1μm).

Figure 5. Functional Imaging of CA1 Pyramidal Neuron

(a) Functional imaging sites selected from a structural image acquired in $3+0D$ scanning mode. *Left*, individual optical sections (scale bars, $50\mu\text{m}$); *right*, user-selected sites on maximum projection image (scale bar, $50\mu\text{m}$). Axial distances from the inherent focal plane of the objective are color coded.

(b) Random access imaging of calcium signals at the selected sites (*top*) during a train of bAPs (*bottom*). Acquisition rate $\sim 10\text{kHz}$.

Figure 6. Fast 3D Monitoring of Dendritic Calcium Dynamics

(a) 3D functional image (using a random access scan) of calcium transients along oblique dendrites during a train of three bAPs ($n = 5$; scale bar $50\mu\text{m}$). Acquisition rate $\sim 3\text{kHz}$.

(b) 3D functional image (using a random access scan) of a portion of an apical dendrite, ($n = 5$; scale bar $25\mu\text{m}$). Acquisition rate $\sim 5\text{kHz}$. Inset, 3D reconstruction. Red and green lines indicate sites that are not easily distinguishable on the maximum projection image since they are located above a brighter dendritic segment.

(c) 3D functional image (using a random access scan) of a zoomed-in portion of an oblique dendrite demonstrating calcium transients in the dendrite as well as in a spine (circled; $n=5$; scale bar $2\mu\text{m}$). Acquisition rate $\sim 3\text{kHz}$.

For all images, axial distances of the user-selected sites are color coded (measured in microns relative to the inherent objective lens focus). All values rounded to nearest micron.