Scanless volumetric imaging by selective access multifocal multiphoton microscopy

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Simultaneous, high-resolution imaging across a large number of synaptic and dendritic sites is critical for understanding how neurons receive and integrate signals. Yet, functional imaging that targets a large number of submicrometer-sized synaptic and dendritic locations poses significant technical challenges. We demonstrate a new parallelized approach to address such questions, increasing the signal-to-noise ratio by an order of magnitude compared to previous approaches. This selective access multifocal multiphoton microscopy uses a spatial light modulator to generate multifocal excitation in three dimensions (3D) and a Gaussian–Laguerre phase plate to simultaneously detect fluorescence from these spots throughout the volume. We test the performance of this system by simultaneously recording \( \text{Ca}^{2+} \) dynamics from cultured neurons at 98–118 locations distributed throughout a 3D volume. This is the first demonstration of 3D imaging in a “single shot” and permits synchronized monitoring of signal propagation across multiple different dendrites.

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1. INTRODUCTION

Synaptic transmission between neurons is the most basic unit of information flow in a neural circuit. How the activity of heterogeneous synaptic inputs is integrated within and across its individual dendrites is a key computation for each neuron in the brain. While synaptic integration at a neuronal level, in particular as it relates to excitatory inputs, has been extensively modeled [1–3], to date it has not been possible to simultaneously monitor synaptic activity across the entire dendritic arbor of a neuron in vivo due to the difficulty of monitoring sparsely distributed points across a large three-dimensional (3D) volume at sufficient speed. In the mouse neocortex, pyramidal neuron dendritic arbor can occupy a volume in the range of \( 1 \times 10^6 \mu m^3 \), depending on depth [4], with synaptic sites on the scale of a few micrometers distributed across the arbor [5].

Tackling this class of neurobiological questions requires high-speed methods for detecting activity at synaptic resolution across many distributed sites. Functional synaptic imaging in a living mouse brain further requires imaging at depths of at least 1–200 \( \mu m \), which is best accomplished by multiphoton excitation. Today, the majority of in vivo neurobiological multiphoton imaging studies rely on point excitation with raster scanning [6–8]. To scan the entire volume, one must move the single excitation point very rapidly throughout the volume. The settling time of the scanner, as well as the sampling rates required to accurately detect fluctuations in the \( \text{Ca}^{2+} \) indicator fluorescence, place very strict limits on the available dwell time for each voxel. The image signal-to-noise ratio (SNR) typically suffers due to the short signal integration time at each voxel.

For monitoring events such as dendritic signal propagation, scanning speeds can be improved by using optical devices to steer the excitation beam [9–13]. Yet, the essential problem of SNR remains the same. All existing methods sequentially target the selected locations, which significantly reduces signal integration time and SNR, even when only 10 or so loci are measured. These constraints have restricted previous approaches to image \( \text{Ca}^{2+} \) activity at synaptic resolution to a relatively small field of view (FOV), allowing the tracking of signal propagation across only a few tens of micrometers of dendrite at any given time [9–11,14,15].

Parallelized imaging can improve scanning speed without incurring an SNR penalty by simultaneously exciting and detecting at multiple locations. Assuming sufficient excitation power can be provided, this parallelization improves imaging speed proportional to the degree of parallelization without compromising SNR. Some recent methods in multiphoton parallelized imaging include multifocal excitation [16–19], temporal focusing (TF)
plane or line illumination [20–23], multiple plane simultaneous imaging [24,25], light field microscopy [26,27], and holographic imaging [24,28–31]. Many of these methods are typically used for monitoring neuronal activity across a large brain area at relatively coarse spatial resolution, on the order of several micrometers [22–28,31]. This level of resolution is not sufficient for studying neuronal integration of synaptic signals, which would require imaging with 3D resolution on the submicrometer scale. In addition, dendrites and spines are smaller than somas; thus their fluorescent signal is weaker, requiring new, higher SNR methods.

To address these challenges, we developed a high-resolution, high-SNR method for simultaneous functional imaging called selective access multifocal multiphoton microscopy (saMMM). This method has three advantages over previously developed techniques, such as random access imaging [9–12] and Bessel beam imaging [32,33] [Fig. 1(a)]. First, wavefront shaping [34] with a spatial light modulator (SLM) enables simultaneous generation of multiple Gaussian excitation spots in 3D at submicrometer resolution. Through the use of higher peak energy (micromillijoule level) femtosecond lasers, over a hundred excitation spots can be generated simultaneously. Second, a Gaussian–Laguerre (GL) phase plate in the detection path extends the imaging depth of field (DoF), enabling 3D simultaneous detection on a 2D camera [26,31,35] without axial scanning. Third, saMMM allows imaging in a truly “scanless” manner from a 3D-distributed region of interest (ROI), so functional time traces are strictly simultaneous as compared to rapid sequential scanning among ROIs [9–13] or whole volume imaging [32,33].

To demonstrate saMMM, we monitored Ca^{2+} signals from more than a hundred selected locations along the dendrites of a single cultured neuron. Our results show that the GL phase plate provides a higher out-of-focus SNR than Gaussian spot targeting, improving 3D resolution, and that the time gains from simultaneous excitation and scanless detection allow increased dwell times that further benefit the SNR. In addition, the scanless nature of saMMM provides a simultaneous time stamp at multiple loci, more faithful than can be provided by sequential scanning.

2. METHODS

A. saMMM with Auxiliary Line-Scan TF Microscopy Setup

The setup is illustrated in Fig. 2(a). The two microscopes share one high peak energy femtosecond laser at 1030 nm (Monaco, Coherent Inc., California). The excitation light is split with a polarizing beam splitter with individual intensities regulated with the half-wave plate. The P-polarized beam is phase-modulated by an SLM (PLUTO-NIR-HR phase-only reflective SLM, HOLOEYE Photonics AG, Germany) that is placed in a conjugate position to the back aperture of the objective (XLUMPlanFL, 20 × , 0.95 NA, Olympus), projecting holographic patterns that generate excitation foci defined by the ROI. The incident angle to the SLM is about 8°. A mirror [M3 in Fig. 2(a)] is inserted before the microscope tube lens [L4 in Fig. 2(a)] to direct the light from the SLM to the specimen while blocking the light from the grating. Two-photon excited fluorescence is deflected by a dichroic mirror into the detection path. A GL phase plate (Customer design, manufactured by HOLO/OR Ltd., Israel) is inserted in the Fourier plane of the image for 3D imaging.

For structural imaging using line-scan TF excitation, the mirror [M3 in Fig. 2(a)] is removed before the microscope tube lens [L4 in Fig. 2(a)] to pass the light from the grating to the specimen while a beam block is placed to block the light from the SLM. In the line-scan TF system, the beam is mechanically scanned in the vertical direction by a galvano motor-driven scanning mirror (6350, Cambridge Technology Inc., Massachusetts). The excitation beam is then focused into a line on a diffraction grating (20RG1200-1000-2, 1200 grooves/mm, 50 × 50 × 6 mm, Newport, USA) by a cylindrical lens. The grating, conjugated to the image plane, diffracts the line in the horizontal direction. The saMMM and the line-scan TF systems also share the same tube lens and objective lens. By changing the incident angle to grating and voltage driving the scanning mirror, the FOV can be adjusted. The FOV of our setup is about 200 μm × 200 μm. For volumetric imaging by line-scan TF, a piezo objective-translator (MIPOS 5, Piezosystem jena GmbH, Germany) is used for axial scanning. Line-scan TF and saMMM
share the same sCMOS camera (Prime95B, Photometrics, Arizona) for detection.

B. Rapid Selection of Discrete Targets for Scanning

Using MATLAB, an automatic tracing algorithm is used to select the local maximum of the image for the 2D targeting. For 3D targeting, the automatic tracing is done with neuTube [36], called from the command line within MATLAB. After the automatic tracing, the spots can be edited manually to adjust positions for better alignment, to distribute locations more regularly, and to add or delete spots for a specific sampling goal. For the volume size described here, the structural image tracing and subsequent calculation of the SLM phase mask takes approximately 1 min.

C. Cell Culture Preparation

Cortical neurons from E18 Sprague-Dawley rat embryos were cultured for 14 days on 18 mm glass coverslips in Neurobasal-A media with 2% B27 supplement and 1% Glutamax. On day in vitro (DIV) 7, cultures were infected with 2 × 10^8 genome copies of AAV1.Syn.NES-jRGECO1a.WPRE.SV40 (Janelia GENIE project supplied through Penn Vector Core). On DIV 14, the coverslips were transferred to a custom imaging chamber, and the Neurobasal media was replaced with Tyrode’s solution. A custom-made warming plate was used to maintain the culture at 37°C during imaging.

### 3. RESULTS

#### A. saMMM with Auxiliary Line-Scan TF

Figure 1(b) outlines the experimental flow for generating scanless excitation and detection using the saMMM setup with the auxiliary line-scan TF multiphoton system (see Methods). Since a selective addressing approach requires a coordinate “map” for targeting specific locations, we started with a structural imaging step to map out the dendritic arbor and determine potential ROIs. Accurately locating several hundreds of submicrometer excitation spots on the targeted positions from the 3D structure “map” requires highly precise registration between the TF system and the saMMM. Thus, we integrated the auxiliary line-scan TF multiphoton imaging system within the saMMM setup [Figs. 1(b), 2(a) and Fig. S1 of Supplement 1]. Running the line-scan TF system at 10 fps allows imaging of a 200 × 200 × 10 μm³ volume in about 1 s. The imaging speed of the line-scan TF is faster than point-scanning (PS) methods by 1 to 2 orders of magnitude [37], while spatial resolution of the line-scan TF is similar to standard PS two-photon microscopy (Fig. S1 of Supplement 1) due to spectral and spatial filling of the objective back aperture [20–22,37].

After structural imaging with the line-scan TF, we selected spots along multiple dendrites of the same cell. The x, y, z coordinates of these spots were used as targets for functional imaging by saMMM. The Gaussian point spread function (PSF) achieves...
submicrometer resolution for selected spot targeting [Figs. 2(b), 2(d), and 2(e)]. The lateral size of the GL PSF changes with depth. From top to bottom, the distance between two lobes are: 2.55, 2.02, 0.6, 2.66, and 2.86 μm [Fig. 2(c)]. The main advantage of the GL PSF is the elongation of the DoF to about 15 μm (full width at half-maximum) in our setup [Fig. 2(c)]. This enables out-of-focus fluorescence to be more efficiently captured by the camera, since the Gaussian PSF without GL phase encoding has a much shorter DoF [Figs. 2(b)–2(d)]. Under the same input power and a fixed lateral integration area of (16 μm × 16 μm) dictated by synaptic density, the total intensity of the GL PSF is the same as the Gaussian PSF at the focal point, but higher than the Gaussian PSF at out-of-focus locations [Figs. 2(f) and 2(g)]. Above ±1 μm, GL spots have progressively better SNR as defocusing is increased. This statement holds when shot noise is the dominant noise source and readout noise from the camera is negligible. Another advantage of including the GL phase plate is that the GL PSF encodes different axial positions with different rotation angles [Figs. 2(c) and 2(h)]. In our setup, 1 μm axial difference corresponds to 11.5° ± 2.5° rotation [Fig. 2(h)]. Because the foci positions are known a priori, the depth-encoding properties of the GL phase plate is a nice bonus, but not essential. The ability to improve SNR for out-of-focus targets with the GL phase plate allows us to record signals from a 200 × 200 × 10 μm³ volume with a 2D camera.

B. High-Throughput Neuronal Ca²⁺ Imaging in a Single 2D Plane

As a first demonstration of the utility of the saMMM system, we tested its performance for high-throughput, high-resolution monitoring of Ca²⁺ dynamics in 2D (Fig. 3). Cultured neurons were transfected with the red Ca²⁺ sensor, jRGECO1a. These cells form arbor over a relatively large area on the culture dish but have a relatively short axial volume of only a few dozen micrometers, providing a mostly 2D specimen. A neuron expressing jRGECO1a was first structurally imaged using the line-scan TF system, rapidly auto-traced in MATLAB, and 113 ROIs were selected, targeting multiple dendritic branches and varying distances from the cell soma, as well as different somas in the FOV (see Methods). Ca²⁺ signals were recorded by saMMM at 100 Hz frame rate simultaneously from all spots for 120 s. Figure 3(a) shows the overlap of the structural image obtained with line-scan TF (magenta) with emission at targeted locations after selective excitation by saMMM (green). We selectively show Ca²⁺ signals recorded from every other spot along three dendrites (labeled in blue, red, and black), sorted based on the distance to the soma. This is the first demonstration of simultaneous monitoring of Ca²⁺ dynamics at more than a hundred locations distributed across a large FOV at submicrometer-level resolution and 100 Hz temporal resolution.

C. High-Throughput 3D Neuronal Ca²⁺ Imaging

To extend the utility of saMMM from 2D to 3D, we placed a GL phase plate in the Fourier plane of the detected image [Fig. 2(a)]. Because cultured cells are essentially planar, to demonstrate 3D detection we positioned the cell at different distances of defocus. As in the 2D case, we could achieve 3D targeting and acquisition in one shot. As outlined in Fig. 1, we acquired the 3D structural image of a cultured neuron (200 × 200 × 10 μm³) expressing jRGECO1a using the line-scan TF system, rapidly auto-traced in MATLAB, and selected 98 (ΔZ = 0), 118 (ΔZ = 3 μm), and 99 (ΔZ = 6 μm) ROIs sequentially in a 3D volume. We then simultaneously monitored spontaneous Ca²⁺ signals from...
these foci, recorded at 100 Hz (ΔZ = 0) and 50 Hz (ΔZ = 3 μm and ΔZ = 6 μm), with and without the GL phase plate (Fig. 4). For defocused recording, 50 Hz frame rate gives better SNR than recording at 100 Hz. In Fig. 4(b), we show representative Ca²⁺ traces for individual spots marked by arrows in Fig. 4(a). When the foci are in focus, Gaussian and GL spots both show a clear Ca²⁺ signal. When the foci are far out of focus, with the GL phase plate the Ca²⁺ signal can still be detected thanks to the elongated DoF, while Gaussian spots are barely detectable. These results demonstrate that with the GL phase plate, Ca²⁺ signals for individual foci are detectable within an axial range from −6 to 6 μm.

To quantitatively compare the SNR of Ca²⁺ signals recorded from Gaussian foci versus GL foci, we defined the “signal” as the peak ΔF/F value, where ΔF and F are the change in fluorescence and the steady-state fluorescence observed at a given dendritic location, respectively. We further defined the “noise” as the standard deviation of ΔF/F in the absence of Ca²⁺ events. For statistical analysis, we repeated this calculation for about 20 spots. For each spot, both signal and noise were averaged from multiple firings [Fig. 4(c)]. The absolute value of SNR is related to the jRGECO1a expression level in the neuron, so it varies from cell to cell.

However, the relative SNR between Gaussian foci and GL foci show the advantage of GL. When the foci are in focus, ΔF/F recorded by Gaussian and GL foci have similar SNR (median of Gaussian, 2.39; median of GL, 2.34). As the foci become more defocused, the extended DoF of the GL foci gradually provides a higher SNR advantage as compared to Gaussian foci. At 3 μm defocus, the GL foci already have better SNR than Gaussian foci (median of Gaussian, 2.38; median of GL, 3.16), and the advantage of GL becomes more obvious at 6 μm defocus (median of Gaussian, 2.02; median of GL, 3.26). These results match the intensity comparison between the Gaussian and GL PSFs in Figs. 2(f)–2(g).

We also evaluated the thermal damage during our experiments. Thermal damage in living systems when using high-energy lasers is the greatest limitation and often constrains the amount of laser power than can be used for excitation [38,39]. For cultured cells, we illuminated a single cell with 100–160 mW (0.8 mW per spot, in total, 125–200 spots) for 30 min (Supplement 1, Fig. S2). The cell was partially photobleached but still firing after 30 min illumination. This shows that even with several hundred foci targeted per neuron, the thermal damage is negligible.
This experiment demonstrates that saMMM can perform 3D volumetric functional imaging “at once.” In the case of Gaussian spots, the DoF covers about 1.8 μm, while for GL spots, the DoF is extended to about 15 μm. The saMMM records Ca\(^{2+}\) signal from all locations simultaneously, so the temporal information obtained across all locations is not staggered, as would be the case for sequential scanning. Because of the parallelization, for the same voxel resident time saMMM affords about 35-fold improvement in SNR as compared to traditional PS.

### D. Improvements in SNR Using saMMM

The SNR is equal to the square root of signal photons when shot noise dominates. For saMMM, the SNR is \(\sqrt{M \cdot R \cdot L}\) times higher than that of PS two-photon microscopy, in which \(M\) is the number of foci, \(R\) is ratio of the voxels in a volume and the number of voxels to be monitored corresponding to synaptic locations and branch points, and \(L\) is a parameter characterizing the relative two-photon excited fluorescence generation efficiency of the laser sources compared with standard titanium-sapphire oscillators used in PS systems. Among these parameters, \(M\) and \(R\) are related to imaging strategy, and \(L\) is mostly related to laser technology.

The SNR improvement of saMMM comes mainly from \(M\) and \(R\). However, future improvements in lasers could enable much higher SNR compared to traditional systems. In a 200 × 200 × 10 μm\(^3\) volume containing a single neuron, the total volume of ROIs, including synapses and branch points, is about 500 μm\(^3\). Thus, compared to exhaustive sampling methods, \(R\) is the ratio between the 200 × 200 × 10 μm\(^3\) sampling amount and the 500 μm\(^3\) ROIs, typically about 800. \(L\) is related to the ratio of repetition rate and pulse width. The regenerative amplifier used in the saMMM system has a slower repetition rate and broader pulse width (1 MHz and 200 fs) than typical titanium-sapphire oscillators (80 MHz and 50 fs). Therefore, fluorescence generation for single-excitation foci is less efficient in the saMMM system by a factor of 320 (\(L\)), given the same optimal pulse energy for the fluorophore. This parameter is limited by current laser technology. With a dispersion compensation unit, it would be possible to achieve a narrower pulse width below 50 fs, potentially improving \(L\) by a factor of 4 compared to the current version of saMMM. Thus saMMM could potentially (saMMMp) be further improved to double the SNR.

According to the SNR equation \(\sqrt{M \cdot R \cdot L}\) and the parameters above, comparing saMMM with standard PS, our approach currently has an SNR advantage of 35-fold in the 3D case. After compressing the pulse width of saMMM, the SNR of saMMMp has an advantage of 70-fold in the 3D case. As compared to saMMM, other competitive methods that employ single-spot random access scanning (RAS) [9–12], suffer from higher \(M\) values due to the lack of parallel excitation, on the order of 500-fold. With these methods, \(L\) is decreased on the order of 320 relative to saMMM, while \(R\) is the same. Thus, saMMM improves the SNR by 1.25 times, and saMMMp improves the SNR by 2.5 times over RAS.

For Bessel beam scanning (BBS), the DoF under NA = 0.93 is 11–20 μm [32,33], which is similar to the DoF of saMMM GL foci. The Bessel beam approach effectively generates a column of synchronous excitation at each axial location and scans them exhaustively across one plane, which does not need to have the “map” at the beginning. As compared to BBS, the saMMM system improves \(R\) by 800 and \(M\) by 50, while decreasing \(L\) by 320. Thus, saMMM improves the SNR by 12 times, and saMMMp improves the SNR by 24 times over BBS. A summary comparing the SNR of saMMM and saMMMp with other prior approaches is presented in Table 1.

### 4. DISCUSSION

In this work, we demonstrate scanless excitation and detection by saMMM that can monitor spontaneous Ca\(^{2+}\) signals in cultured neurons at more than a hundred locations in a 3D volume. Our selective access approach applies an SLM to flexibly control the excitation positions, combined with a GL phase plate to elongate the DoF so that a single frame image collects signals across a 10 μm thick volume. This approach significantly improves the SNR and volumetric imaging speed across an FOV of hundreds of micrometers retaining submicrometer spatial resolution.

We expect that future developments in saMMM will allow higher speed volumetric imaging with even higher SNR. First, SNR always improves with further parallelization. The SLM could generate more spots in the current FOV, leading to a larger \(M\) value. Two factors currently limit the total number of spots that can be generated holographically. First, with limited laser output, the pulse energy available at each spot drops with increasing number. Second, the diffraction efficiency of the SLM decreases when increasing the number of spots [29]. In our experiment, hundreds of spots can be generated with good efficiency. For generating more diffraction spots, an SLM with more pixels would probably be helpful. In the 2D case, the SLM phase pattern is exactly the 3D projection of an Ewald sphere [34,40], which is exactly the 3D Fourier transform of the targeted spots. In the 3D case, the SLM phase pattern is the parallel projection of the 3D volume containing a single neuron, the total volume of micrometers retaining submicrometer spatial resolution.

We also show that saMMM can be used to monitor Ca\(^{2+}\) activity in cultured neurons that have limited axial depth. To explore how tissue scattering influences saMMM, we measured the GL PSF inside a 50 μm mouse brain slice (Fig. S3 of Supplement 1). We found that tissue aberration and scattering barely influences the extended DoF and the lateral size of the GL PSF. Also, tissue scattering was not significant within an imaging depth on the order of 1–2 mean free path (MFP) [7]. For deeper imaging (several MFPs), adaptive optics (AO) could be used for aberration correction [41]. Thus, with minor modifications, saMMM may potentially be suitable for larger-volume, lower-resolution studies inside tissues.

| Table 1. Comparison of saMMM with Other Methods |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | PS              | BBS             | RAS             | saMMM           | saMMMp          |
| \(R\)          | 1               | 1               | 800             | 800             | 800             |
| \(M\)          | 1               | 10              | 1               | 500             | 500             |
| \(L\)          | 1               | 1               | 1               | 1/320           | 1/80            |
| SNR            | 1               | 3               | 28              | 35              | 70              |

To summarize, the saM MM realizes simultaneous excitation and detection of fluorescence in a 3D volume. We demonstrate greatly improved SNR of functional imaging by parallelization of over a hundred foci with selective addressing. This selective targeting method improves functional imaging speed while reducing photodamage and can potentially be applied to 3D high-throughput imaging and monitoring of local activities across entire neurons within a living animal.

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See Supplement 1 for supporting content.

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