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# High-speed two-photon microscopy with adaptive line-excitation

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**We present a two-photon fluorescence microscope designed for high-speed imaging of neural activity at cellular resolution. Our microscope uses an adaptive sampling scheme with line illumination. Instead of building images pixel by pixel via scanning a diffraction-limited spot across the sample, our scheme only illuminates the regions of interest (i.e., neuronal cell bodies) and samples a large area of them in a single measurement. Such a scheme significantly increases the imaging speed and reduces the overall laser power on the brain tissue. Using this approach, we performed high-speed imaging of the neuronal activity in mouse cortex *in vivo*. Our method provides a sampling strategy in laser-scanning two-photon microscopy and will be powerful for high-throughput imaging of neural activity.** © 2024 Optica Publishing Group under the terms of the [Optica Open Access Publishing Agreement](#)

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## 1. INTRODUCTION

Two-photon laser scanning microscopy can image deep into the tissue with a high signal-to-background ratio, tight axial confinement, and low phototoxicity [1–3]. When used in conjunction with functional fluorescent indicators, two-photon microscopy provides a powerful tool to monitor neuronal signals and activity [4–7]. Conventional two-photon microscopes build the image by raster scanning a laser focus point by point on the sample, resulting in a tradeoff between sampling speed, spatial resolution, and field of view (FOV). When imaging a sample with a large FOV at fine spatial resolution, the temporal resolution could become poor, which may not faithfully capture the neuronal signals. The single-beam scanning strategy thus limits the imaging throughput. Another limitation of conventional two-photon microscopes is the blind scanning strategy. In particular, it is highly inefficient to record dynamic signals where the sample is repeatedly imaged in time. It not only wastes time imaging areas without useful information, but it also deposits unnecessary heat and induces possible damage to the sample.

Various types of beam multiplexing techniques [8–16] have been proposed and demonstrated to alleviate the tradeoff between the spatiotemporal resolution and FOV and to increase the imaging throughput. In these approaches, multiple beams are used to image the sample, each scanning a sub-FOV. The signals are then demixed and assigned to the proper pixels in the whole image. While effective, the imaging throughput is still limited by the number of beams that can be multiplexed and the demixing quality.

Furthermore, as the imaging throughput increases, the overall laser power on the tissue will inevitably increase. This induces excessive heat on the brain tissue and can cause tissue damage [17].

Random-access two-photon microscopes realized by acousto-optic deflectors (AODs) [18–20] can overcome the limitations of blind scanning strategies and reduce heating of the brain tissue. Instead of raster scanning, AODs rapidly steer the beam in the imaging process to select the desirable regions of interest (ROIs), such as the neuronal cell bodies. We term this type of sampling strategy adaptive sampling, as the sampling location is tailored to the ROIs on the tissue. While AODs have an increased imaging speed and reduced photodamage on the tissue, they are generally expensive, sensitive to excitation wavelength, and have a limited scanning angle and thus FOV. Another strategy for adaptive sampling utilizes raster scanning with a temporally modulated laser source [21]. However, it is incompatible with beam multiplexing schemes and has a limited imaging throughput.

We report a new adaptive sampling strategy for two-photon microscopes with high imaging speed/throughput and low laser power on the brain tissue. This microscope is optimized for population calcium imaging, where the ROIs are the neuronal cell bodies. Instead of using a diffraction-limited spot, we image the sample by scanning a short excitation line. Furthermore, we spatially modulate the line pattern so only the neuronal cell bodies are excited. Our scheme significantly increases the imaging speed as it images a large portion of the tissue in a single measurement and thus reduces the total amount of measurements per frame. The

imaging process itself pre-processes the data otherwise recorded by the point scanning approach, as it sums up the pixels within the short-excitation line, which mostly belong to a single ROI. The number of pixels in the recording is considerably reduced, which could reduce the storage and alleviate the demand for the required computational resources to process the data (i.e., segmentation and temporal traces demixing and extraction). Finally, by only illuminating the ROIs, it noticeably reduces the laser power on the tissue. Using this microscope, we performed high-speed calcium imaging of mouse cortex *in vivo*. Compared to the typical two-photon microscope using the point scanning strategy, we increased the imaging speed by  $\sim 5 - 10\times$  and reduced the laser power on the brain by  $> 10\times$ . Our new adaptive sampling strategy is compatible with beam multiplexing techniques and holds great promise to significantly enhance the imaging throughput capabilities of two-photon microscopy.

## 2. PRINCIPLE OF ADAPTIVE LINE-EXCITATION

In our adaptive sampling scheme, the brain tissue is illuminated with a short line that is dynamically patterned to match the local structure of the ROIs. Scanning such a spatially modulated excitation line over the tissue allows us to capture the ROIs but not the background area in the image plane [Figs. 1(a)–1(c)]. The spatial modulation of the excitation line is realized by a digital micromirror device (DMD), which has been widely used to shape the illumination beams through its intensity modulation or indirect phase modulation via a Lee hologram [22] in two-photon systems, for applications in imaging [23–25], micro-fabrication [26], and optogenetics [27]. Here, the DMD functions as an intensity modulator and is loaded with a binary mask that matches the morphology of the neuronal cell bodies (i.e., ROIs) on the sample plane. The binary mask contains the spatial footprint of the ROIs, obtained through a calibration process where we image the sample plane in high resolution, followed by segmentation. The DMD is placed at a conjugate plane of the sample plane. We shape the laser beam (femtosecond laser at 920 nm) into a line and scan it across the DMD through a resonant scanner (8 kHz) and galvanometer mirror. The deflected light from the DMD is then spatially modulated to carry the pertinent information of the ROIs, which is optically relayed to the sample plane to image the brain tissue [Fig. 1(d), Supplement 1, Notes 1–2]. The pattern on the DMD is obtained by a calibration process, which performs a high-resolution recording of the sample, equivalent to the point-by-point scanning scheme. This can be conducted in the same microscope by merely configuring the DMD pattern so the ON pixels form periodic rows (Supplement 1, Note 3, Supplement 1, Figs. S3–S4), without any physical change of the optical system. This ensures an optimal alignment and calibration for pattern registration in adaptive illumination.

In addition to spatial light modulator, the DMD also serves as a diffraction grating, which could create the temporal focusing effect [28–33] and thus tighten the axial confinement of the point-spread function on the sample plane (Supplement 1, Note 4, Supplement 1, Fig. S7). The DMD is configured to be at blazed condition and positioned such that the chief ray (corresponding to the central wavelength of the laser light) of the diffraction beam at the blazed condition propagates on axis to the subsequent optics after the DMD (Supplement 1, Note 2, Supplement 1, Fig. S1). As a result, the DMD surface is not parallel with the scan lens but is tilted to fulfill the blazed angle condition. In this way, the

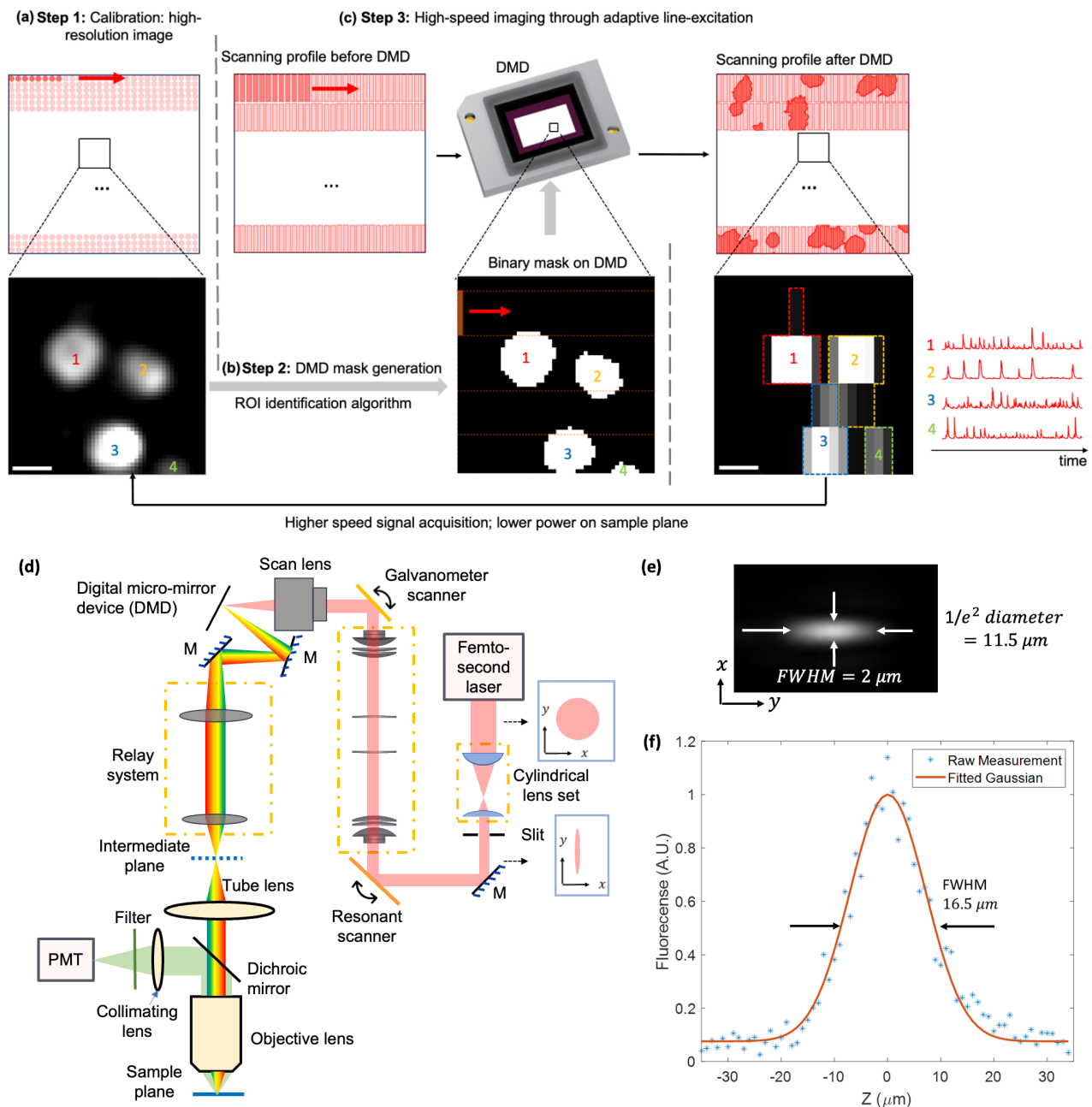
spatial focusing plane of the scan lens is mismatched with the temporal focusing plane (i.e., the DMD surface) while the beam is being scanned. To resolve this, we deliberately introduced an in-plane decentering spatial displacement of the scan lens [34] (Supplement 1, Note 2, Supplement 1, Fig. S2). This ensures the overlap of the spatial focusing plane and temporal focusing plane and a consistent pattern of the incident line illumination across the entire surface of the DMD.

Compared to the conventional point-by-point blind sampling scheme, our method has two distinct features. First, we sample a larger region in a single measurement. This reduces the number of rows in the imaging and thus increases the frame rate. In our demonstration, we shaped the excitation line to be  $\sim 11.5\ \mu\text{m}$  in length ( $1/e^2$ ) on the sample plane [Fig. 1(e)], which is similar to the diameter of the neuronal cell body in a mouse brain ( $10\text{--}15\ \mu\text{m}$ ). This allows a fast imaging speed while maintaining cellular resolution and avoiding excessive signal crosstalk between adjacent neurons in a single measurement, as most of the measurements would contain information about only a single neuron in a typical labeling condition. Furthermore, our method effectively pre-processes the images otherwise recorded by the blind sampling approach, as we condense the pixels, which mostly belong to a single source, to a single pixel. This not only saves the memory to store the raw images but also reduces the data volume and, thus, the computation time in the subsequent data processing (segmentation and extraction of the temporal activity traces). Signal demixing could be processed post hoc, with prior knowledge of all the source locations. This could resolve the signal crosstalk in the scenarios when a single measurement contains information from more than one source. Second, the beam modulation from the DMD enables an exclusive sampling of the neuronal cell bodies and avoids the unnecessary excitation of background regions. It greatly reduces the overall laser power delivered to the brain tissue and, thus, the thermal damage. Though our excitation pattern was a line, it was thin ( $\sim 2.0\ \mu\text{m}$ , full-width-at-half-maximum, FWHM) [Fig. 1(e)]. The axial FWHM of the PSF was measured to be  $\sim 16.5\ \mu\text{m}$  [Fig. 1(f)].

## 3. RESULTS

### A. Simulations of the Adaptive Line-Excitation Sampling Process in Neuronal Imaging

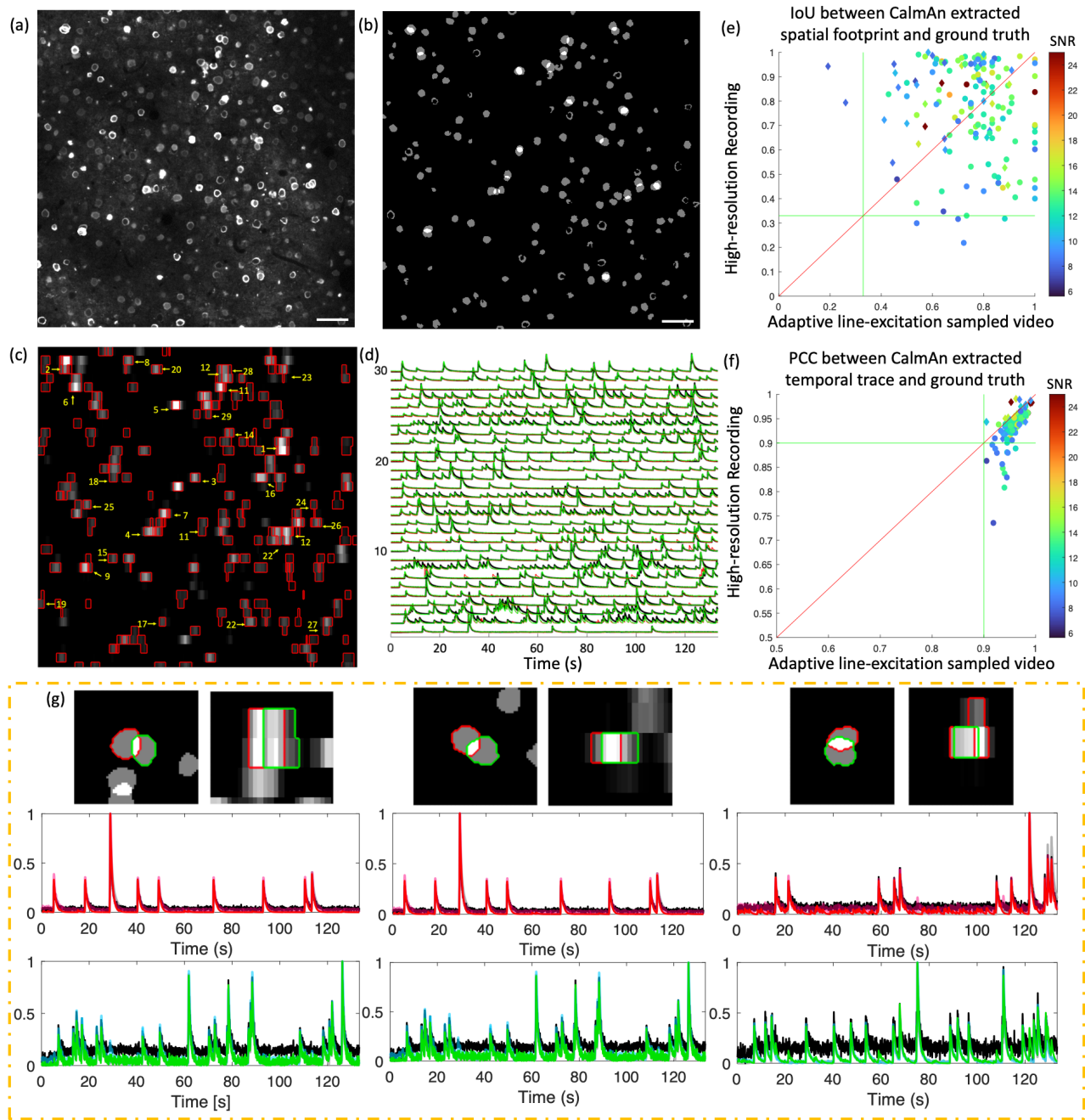
We simulated the adaptive line-excitation sampling process in a simulated calcium imaging dataset and investigated whether the signal from individual neurons, including those having spatial overlap with each other, could be faithfully extracted (Fig. 2, Supplement 1, Fig. S8). We generated a simulated calcium recording [Fig. 2(a),  $500\ \mu\text{m} \times 500\ \mu\text{m}$ ,  $500 \times 500$  pixels, 2000 frames] [35] with GCaMP6f at 15 Hz frame rate, with 80% Poisson noise and 80% Gaussian noise added. We generated a binary mask from the spatial footprint of the neurons [Fig. 2(b)] and created the adaptive-line sampled videos from a bi-directional scanning trajectory on the original high-resolution recording. We then used CalmAn, a constrained non-negative matrix factorization (CNMF) algorithm [36,37], with the prior knowledge of the neuronal locations from the binary mask, to extract the neuronal spatial footprint [Fig. 2(c)] and temporal activity traces [Fig. 2(d)] in the adaptive-line sampled recording. Though the number of pixels in individual neurons in the adaptive-line sampled recordings is small, CalmAn successfully segmented the spatial footprint



**Fig. 1.** Principle, optical setup, and point-spread function (PSF) of the two-photon scanning microscope with adaptive line-excitation. (a)–(c) Working principles of adaptive line-excitation. (a) A high-resolution video of the neuronal activity is acquired in the calibration step through an equivalent point scanning strategy. (b) The ROIs (i.e., neuronal cell bodies) could then be segmented. They are then binarized into a mask and loaded to the DMD. The laser (920 nm femtosecond laser) light is first shaped to a short line and incident to the DMD, which is located at the conjugate plane of the sample plane and works as an intensity spatial modulator. (c) The beam diffracted from the DMD carries the information of the ROI, and illuminates the corresponding part of the sample ROI. Only the ROIs but not the background region are imaged. Top, illustration of the excitation scheme on the sample, with the arrow showing the beam scanning direction. Bottom, (a)(c) zoom-in view of a sub-region of the image recorded by the photomultiplier tube (PMT). (b) Binary mask loaded on the DMD. The four ROIs are labeled in different numbers. (d) Schematic of the two-photon microscope setup with adaptive line-excitation scheme. The laser beam is first shaped into a short line, which is scanned by a resonant scanner and a galvanometer mirror onto the DMD. Light diffracted from the DMD is relayed to the sample plane through a relay system and the tube lens and objective lens. The fluorescence is detected by the PMT. M, mirror. (e) Measured PSF in the lateral direction ( $xy$ ) for line-excitation. (f) Measured axial PSF using  $5 \mu\text{m}$  fluorescent beads. Scale bar in (a) and (c) is  $10 \mu\text{m}$ .

of the neurons with a high intersection over union (IoU) values against the reference spatial footprint calculated from the binary mask and scanning trajectory [Fig. 2(e)] and extracted the temporal traces with high Pearson correlation coefficients (PCCs) against the ground truth [Fig. 2(f)]. Crucially, the neuronal signals

could be generally demixed from neurons with overlapping spatial footprints [Fig. 2(g)]. There are a small portion of ROIs that cannot be demixed well due to their strong spatial overlap or weak signal-to-noise ratio in the non-overlap regions, which is a general challenge of CNMF (Supplement 1, Fig. S8). We note that both



**Fig. 2.** Simulation of the adaptive line-excitation sampling in calcium imaging. (a) Time-series standard-derivation projection frame of the simulated high-resolution calcium imaging video. (b) Mask for (a) showing individual ROIs, constructed from the ground truth in simulation. Some of the neurons have spatial overlaps with others. (c) Time-series average projection frame of the video constructed by the adaptive line-excitation sampling on the original high-resolution video, using the mask shown in (b). The frame was resized to the same dimension as the original video. The spatial footprints of the extracted ROIs through CalmAn are outlined in red. (d) Extracted normalized temporal activity traces of the representative neurons in (c), which were indicated by the yellow arrows in (c). Black, ground truth with noise and background included; green, extracted traces using CalmAn on the original high-resolution video; red, extracted traces using CalmAn on the adaptive line-excitation sampled video. (e) Intersection over union (IoU) between the CalmAn-extracted spatial footprint and the ground truth of individual neurons in the high-resolution video versus that calculated for the adaptive line-excitation sampled video. The green lines are indications of  $\text{IoU} = 0.33$ . The color of each dot shows the signal-to-noise ratio (SNR) of individual neurons in the original high-resolution video. The SNR is defined as the maximum of the denoised temporal signal (with signal baseline included) over the standard deviation of the noise. The ROIs with spatial overlap with others are indicated by diamond shape symbols, while the isolated ROIs are plotted in circular dots. (f) Same as (e), but for the Pearson correlation coefficient (PCC) between the CalmAn-extracted traces and the noise-free ground truth traces. The green lines are indications of  $\text{PCC} = 0.9$ . (g) Three pairs of comparisons illustrating that the neuronal signals from the neurons with spatial overlaps could be demixed in the adaptive line-excitation sampled video. In each pair group, the top-left and top-right figures show the spatial footprint of the neurons in the high-resolution ground truth and the time-series average projection frame of adaptive line-excitation sampled video, respectively. The middle panel shows the temporal traces of the ROI contoured in red: gray, ground truth without noise and background; black, ground truth with noise and background included; red, CalmAn-extracted from the adaptive line-excitation sampled video; magenta, CalmAn-extracted from the high-resolution video. Similarly, the bottom panel shows the temporal traces of ROI contoured in green: gray, ground truth without noise and background; black, ground truth with noise and background included; green, CalmAn-extracted from the adaptive line-excitation sampled video; cyan, CalmAn-extracted from the high-resolution video. All the traces are normalized to  $[0, 1]$ . Scale bar in (a) and (b) is  $50 \mu\text{m}$ .

the IoU and the PCC values in the adaptive-line sampled recording do not differ significantly from the CalmAn results on the original high-resolution recording ( $p = 0.32$  for IoU,  $p = 0.54$  for PCC, paired t-test). Overall, our results illustrated that the adaptive-line sampled recording, though significantly down-sampled in one direction, preserved the information as the original high-resolution recording and could be processed through calcium imaging processing tools such as CalmAn, and with significantly reduced computational resources because of the reduced number of pixels in the video.

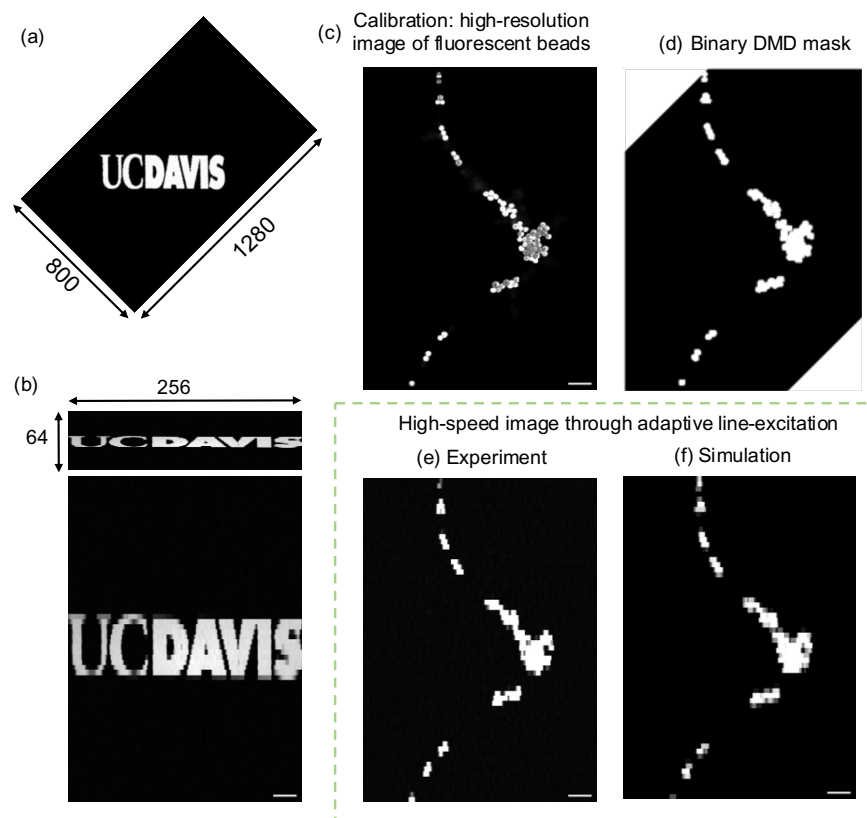
## B. Validation of Adaptive Illumination Through Phantom Samples

We validated the beam patterning capability of the DMD and the concept of adaptive sampling through fluorescent phantom samples (Fig. 3). Using a uniform fluorescent slab, we first assessed the encoding capabilities of our system in defining arbitrary binary masks and projecting the desired patterns onto the sample plane [Figs. 3(a) and 3(b)]. The image recorded by the PMT matched well with the binary mask loaded on the DMD, confirming the conjugate relationship between the DMD and sample plane. We then validated that the binary pattern on the DMD, when projected to the sample, could indeed overlap with the ROIs on the sample structures [Figs. 3(c)–3(f)]. Here, we used a phantom

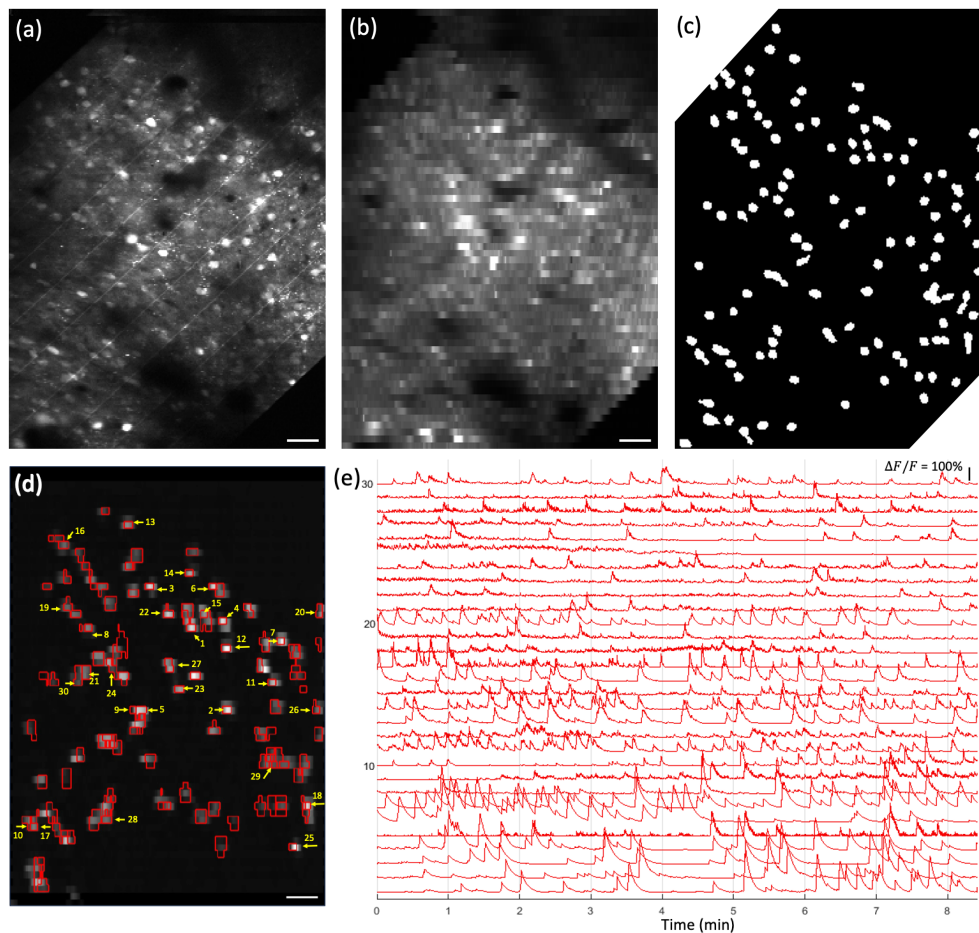
sample composed of randomly distributed fluorescent beads with a diameter of  $12\ \mu\text{m}$ . Through a calibration process that is equivalent to point scanning (Supplement 1, Note 3, Supplement 1, Figs. S3–S4), we obtained a high-resolution image of the sample with  $256 \times 320$  pixels, in a resolution of  $\sim 2.0\ \mu\text{m} \times 2.2\ \mu\text{m}$  [Fig. 3(c)]. Such a calibration process mapped individual DMD pixels to the sample coordinates. We then transformed the high-resolution image to the DMD mask [Fig. 3(d)] and performed the high-speed line scanning with adaptive sampling. The acquired image matched well with the DMD mask, verifying that the mask aligned well with the sample plane [Fig. 3(e)]. Finally, we simulated the high-speed scanning results based on the high-resolution image [Fig. 3(c)] and the bi-directional scanning trajectory. The simulated image [Fig. 3(f)] is highly similar to the experimental result [Fig. 3(e)], further verifying the robust mapping relationship between the DMD and the sample structure.

## C. High-Speed Calcium Imaging of Neuronal Activity In Vivo

We conducted *in vivo* experiments to monitor the cortical activity of layer 2/3 in the primary visual cortex (V1) in an awake mouse transfected with calcium indicators GCaMP6f [38] (Supplement 1, Note 7), over an FOV of  $\sim 500\ \mu\text{m} \times 695\ \mu\text{m}$ . Following the procedure outlined in Figs. 1(a)–1(c), we first



**Fig. 3.** Validation of the adaptive sampling scheme through phantom samples. (a),(b) Projection of the DMD mask onto a uniform fluorescent slab through adaptive short-line excitation. (a) DMD mask ( $1280 \times 800$  pixels) with characters “UC DAVIS.” (b) Top: raw image ( $256 \times 64$  pixels) acquired from PMT through adaptive line-excitation with bi-directional scanning; bottom: interpolated and resized image ( $500 \times 695$  pixels) with square pixels. (c)–(f) Imaging of a phantom sample with randomly distributed fluorescent beads, in  $12\ \mu\text{m}$  diameters. (c) A high-resolution image of the sample was acquired through the equivalent point scanning approach. (d) Binary mask on the DMD. The two corner regions of the mask were outside the DMD active regions and were not displayed. (e) Single frame of the recording from the sample acquired at 198 Hz, using the adaptive line-excitation with bi-directional scanning. (f) Simulated high-speed image based on the binary mask and the bi-directional scanning trajectory of the resonant scanner. Scale bar in (b),(c),(e),(f) is  $50\ \mu\text{m}$ .



**Fig. 4.** *In vivo* calcium imaging of mouse V1 at 150  $\mu\text{m}$  depth using the adaptive line-excitation two-photon microscope. (a) Time-series average projection frame of the high-resolution image from the effective point scanning method, which includes the fine contours of the neurons and the background. The image has  $256 \times 320$  pixels over a  $500 \mu\text{m} \times 695 \mu\text{m}$  FOV. The periodic lines were artifacts and formed due to the tilted scanning trajectory and corresponding shifted DMD pixels (Supplement 1, Note 3, Supplement 1, Fig. S6). They did not impact the segmentation results. (b) Time-series average projection frame of the line-scanning sampled video ( $256 \times 64$  pixels) for the same FOV in (a) without adaptive sampling, where all the pixels in the DMD were turned on. (c) Corresponding binary mask for (a), constructed from the ROI segmentation algorithm of SUNS. (d) Time-series average projection frame of the line-scanning sampled video ( $256 \times 64$  pixels) with adaptive sampling, by applying the mask on DMD. Only the ROIs defined in (c) were illuminated and sampled. The spatial footprints of the extracted ROIs through CalmAn are outlined in red. (e) Temporal activity traces of the representative neurons recorded at 198 Hz, which were indicated by the yellow arrows in (d). The temporal activity traces were extracted by CalmAn. Scale bar in (a),(b),(d) is 50  $\mu\text{m}$ .

calibrated the spatial location of the ROIs by obtaining the high-resolution recording of the sample plane [Fig. 4(a),  $256 \times 320$  pixels, in a resolution of  $2.0 \mu\text{m} \times 2.2 \mu\text{m}$ ] at a depth of 150  $\mu\text{m}$  and segmenting the recording using SUNS [39] [Fig. 4(c), Supplement 1, Note 6], a state-of-the-art fast segmentation algorithm on calcium imaging. In the represented example, we found 130 ROIs, which were the active neurons during the video acquisition period in calibration ( $\sim 5$  min, Supplement 1, Note 3). With the spatial footprints of these ROIs displayed on the DMD, we conducted high-speed recording through adaptive line-excitation [Fig. 4(d),  $256 \times 64$  pixels]. Notably, our sampling strategy reduced the number of rows scanned by the resonant scanner and thus increased the frame rate. With the height of each row being  $\sim 11 \mu\text{m}$ , and using a bi-directional scanning scheme, we achieved a frame rate of 198 Hz over the FOV  $\sim 500 \mu\text{m} \times 695 \mu\text{m}$ . Such a frame rate is significantly higher than the typical ones in conventional two-photon microscopes using the point scanning strategy. Compared to the case where only line-excitation was used but

without the adaptive sampling strategy [Fig. 4(b), with the binary mask on the DMD being all “1” by turning on all DMD pixels], the adaptively sampled images [Fig. 4(d)] used a significantly smaller average laser power on sample ( $\sim 14\times$  smaller than the case without adaptive sampling, as the occupied area of ROIs over the entire plane is  $\sim 7.25\%$ ). In our case of adaptive line-excitation, only  $\sim 1.5$  mW of average laser power was delivered to the brain tissue, which was a considerable drop to avoid thermal damage to the mouse brain. The non-ROI background regions appear to be dark in Fig. 4(d) as they were not imaged, which could facilitate the subsequent ROI segmentation and temporal trace extraction. Finally, from the high-speed recording with the adaptive line-excitation strategy, we extracted the temporal activity traces of individual ROIs through CalmAn [36,37] [Fig. 4(e)]. In the CalmAn processing, as we had prior knowledge of the pixel locations of individual ROIs, we used their centroid pixels to initialize the search for their spatial footprint (Supplement 1, Note 6). This increased the efficiency and efficacy of the algorithm. We further validated the

system's capabilities by imaging the cortex at a depth of 450  $\mu\text{m}$ , where we successfully extracted 91 ROIs (Supplement 1, Fig. S5).

#### 4. DISCUSSION

In summary, we proposed and demonstrated the concept of adaptive sampling with line illumination in two-photon microscopy. The DMD functions as an amplitude spatial light modulator as well as a blazed grating for temporal focusing. By using line illumination and only exciting the regions of interest rather than the entire field of view, we could achieve a high imaging speed (up to 198 Hz with 500  $\mu\text{m} \times 695 \mu\text{m}$  FOV) while reducing the overall laser power and thus phototoxicity on the sample. Crucially, our method is compatible with many other beam multiplexing techniques, such as spatiotemporal multiplexing [9,14], so as to further increase the imaging throughput. The capability to reject the illumination on the non-ROI background regions becomes particularly important to reduce the overall laser power and heat generation in the brain tissue when multiple beams scan the tissue. While designed for high-speed imaging, our microscope retains the capability for the equivalent point scanning high-resolution imaging by properly setting and cycling the DMD pattern in synchronization with the scanner and without physically modifying the optical setup, as in our calibration step. Our adaptive sampling microscope setup could be further optimized, as the current PSF (axial FWHM=16.5  $\mu\text{m}$ ) may result in out-of-focus backgrounds when imaging the neuronal cell bodies. Such a background could be eliminated by further tightening the axial extent of the beam (Supplement 1, Note 4). Alternatively, the background signals near the cell bodies could be captured by a proper configuration of the DMD mask. Algorithms such as matrix factorization could then be used to remove the local background from the cell bodies.

The line-scanning approach is one type of PSF engineering used to increase imaging throughput [40]. Here, we used short-line excitation with a single-pixel detector. Two similar strategies involve pairing a long-line excitation with a camera [41] or a long-line excitation with a linear PMT array [42]. They have challenges in imaging deep due to light scattering. Two other long-line-excitation strategies use a single pixel detector and require imaging the sample through multiple line patterns and then reconstructing the image computationally through principles of compressive sensing [43] or topographical imaging [44]. They typically require intense computational resources or complex system setups. Our strategy avoids these challenges. One advantage of our method is that the signal in each measurement over the short-line illumination mostly comes from the same neuronal source. Such a strategy essentially pre-processes the data, and thus reduces the signal processing time as the number of pixels in the image is reduced. If there are multiple sources in a single short line, they could be separated through demixing algorithms such as non-negative matrix factorization (e.g., CalmAn [36,37]). We note that our selective excitation method shares a similar concept with SLAP2 [45,46]. There, instead of using a short-line to perform a two-dimensional raster scanning across the DMD, SLAP2 uses a long-line to scan the DMD in one dimension. If there are multiple ROIs along the line, only pixels for a specific ROI are turned on in the DMD at a time to avoid imaging multiple ROIs together and mixing their signals. The mask of the DMD could then be changed upon the next scan to image another ROI. When the ROIs are highly sparse, the frame rate could be particularly high. However, the effective insertion loss at the DMD is significantly high, necessitating the

use of a high-power laser. In our approach, the length of the line is designed to be comparable to the diameter of the neurons. This results in a substantially reduced effective insertion loss at the DMD and allows one to use a typical 80 MHz femtosecond laser, which is more commonly available and cost-effective.

Previous reports have demonstrated block-scanning [31,32], where the illumination was a 5  $\mu\text{m} \times 5 \mu\text{m}$  block, and their fluorescence was summed together into a single measurement. There, a low-repetition-rate laser was used, and each block was sampled with one laser pulse, so as to avoid oversampling in the fast-scanning axis. The resonant scanner had to synchronize with the laser pulse clock, which required a custom-tuned resonant scanner and complicated electronic controls. Our method uses line illumination, and the linewidth in the fast-scanning axis is thin. Hence, we could use a typical 80 MHz laser without synchronization between the scanner and the laser clock. Furthermore, as the line rate is determined by the resonant frequency of the scanner, short-line scanning could achieve the same speed as block scanning.

Our adaptive sampling strategy uses a DMD to spatially modulate the excitation pattern. Compared to existing adaptive sampling approaches (AODs [18–20] and adaptive laser source [21]), our method requires simpler hardware and is compatible with beam multiplexing. In particular, in contrast to the method using a temporally modulated laser source, we do not need the synchronization of the modulator and the scanner. Like other adaptive sampling approaches, our method requires prior knowledge of the ROIs, and the imaging quality is sensitive to the motion of the tissue. This could be readily resolved by including a real-time feedback loop and compensating the motion through additional scanners [20].

In typical two-photon microscopes, there is a tradeoff between imaging throughput and required laser power on the tissue. Our adaptive line-excitation two-photon microscope alleviates this tradeoff and could simultaneously increase the imaging throughput and reduce the on-tissue laser power. By combining our method with beam multiplexing techniques and using a faster resonant scanner, the imaging speed could be in kilohertz and thus suitable for voltage imaging. Though not demonstrated here, our method is also compatible with three-dimensional/volumetric imaging by incorporating an axial scanning mechanism (such as tunable lenses) after the DMD. This allows for calcium imaging over a large 3D volume. Finally, while we focused on imaging neuronal cell bodies here, we could also optimize our method (i.e., by increasing the numerical aperture of the beam so as to reduce the width and axial extent of the excitation line) to image ROIs with finer features such as dendrites and spines.

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**Disclosures.** The authors declare no conflicts of interest.

**Data availability.** Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

**Supplemental document.** See Supplement 1 for supporting content.



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