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Fast volumetric calcium imaging across multiple cortical layers using sculpted light

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Abstract

While whole-organism calcium imaging in small and semi-transparent animals has been demonstrated, capturing the functional dynamics of large-scale neuronal circuits in awake, behaving mammals at high speed and resolution has remained one of the main frontiers in systems neuroscience. Here we present a novel method based on light sculpting that enables unbiased single and dual-plane high-speed (up to 160 Hz) calcium imaging, as well as *in vivo* volumetric calcium imaging of a mouse cortical column ($0.5 \times 0.5 \times 0.5$ mm) at single-cell resolution and fast

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Author Contributions

R.P. contributed to the conceptualization of the imaging approach, designed and built imaging system, performed experiments, wrote software, analyzed data, and wrote the manuscript. A.J.V. designed and built the custom FCPA system together with A.F. under the guidance of A.B. and performed experiments. A.J.P.A. performed virus injections, cranial window surgeries in hippocampus and cortical areas performed imaging experiments, histology experiments, analyzed data and contributed to the writing of the manuscript. S.W. performed experiments, wrote software, analyzed data and wrote the manuscript. B.S.H. performed the viral injections and cranial window implants for the cortical imaging experiments. B.S.H. and P.G. shared expertise on *in vivo* imaging of awake mice and provided inputs to the final version of the manuscript. T.N. wrote control software and performed *in vivo* characterization and control experiments and provided inputs to the final version of the manuscript. J.D. wrote analysis code and analyzed data. A.V. conceived and led project, designed imaging system and *in vivo* mouse experiments and wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

volume rates (3 – 6 Hz). This is achieved by tailoring the point-spread function of our microscope to the structures of interest, and by maximizing the signal-to-noise ratio by using a home-built fiber laser amplifier and synchronizing its pulses to the imaging voxel speed. This has enabled *in-vivo* recording of calcium dynamics of several thousand neurons across cortical layers and in the hippocampus of awake behaving mice.

Unraveling how sensory information is processed and stored in the complex, highly interconnected mammalian brain requires tools that are capable of recording the activity of all the neurons that comprise a functional network and its individual circuits at single-neuron resolution and over physiologically relevant time scales. Using sensitive and genetically encoded calcium (Ca^{2+}) indicators, such as GCaMP¹ in combination with one-photon excitation coupled with wide-field detection schemes, various high-speed Ca^{2+} -imaging methods have recently been developed on various platforms such as light-sheet microscopy^{2–4}, light-field microscopy^{5,6} and swept confocally aligned planar excitation⁷. Despite the ability to record from many neurons in parallel, the performance of these wide-field detection schemes rapidly degrades with imaging depth due to light scattering, thereby restricting their utility to the imaging of thin or weakly scattering samples such as *C. elegans*^{4,6}, larval zebrafish^{6,8} or relatively superficial brain areas in rodents⁷.

In tissue with higher light scattering, such as the rodent brain, two-photon scanning microscopy (2PM)⁹ provides a robust method for avoiding the effects of scattering¹⁰ due to its ability to effectively reduce out-of-focus fluorescence, and has emerged as the method of choice for high-resolution brain imaging at greater depths. However, conventional 2PM, which utilizes diffraction-limited excitation spots, suffers from low temporal resolution. This is because even in the absence of any hardware limitations for scanning and laser sources the obtainable signal from each voxel is limited by the fluorescence lifetime. Since the inception of two-photon microscopy, there have been a number of efforts to increase the imaging field-of-view (FOV), speed and the obtainable acquisition depth^{11,12}. These improvements include extended depth of field excitation¹³, remote axial scanning strategies¹⁴ and the use of electro-tunable (ETLs)¹⁵ or ultrasound lenses^{16,17} for fast focusing. Other fast sequential imaging strategies are based on acousto-optic deflectors (AODs) and only excite and detect from a limited set of diffraction limited points on neurons at high speed within a relatively large volume of tissue ($\sim 1 \text{ mm}^3$). However, while such 3D AOD-based systems provide high sampling speeds at isolated points within the sample^{18–20}, they typically require *a-priori* knowledge of neuronal positions and are typically prone to errors from sample movement. Other approaches have utilized spatial²¹ and temporal^{22,23} multiplexing strategies to simultaneously image from several laterally or axially separated planes, in some cases in combination with holography for excitation, and signal demixing during post-processing of the recorded signals^{24,25}. However, these approaches are currently restricted to a few planes at a time and would be eventually limited by signal statistics and crosstalk, i.e. lack of sparsity of neuronal activity in space and time. An overview of the typical performance of some of these state-of-the-art multi-photon calcium imaging techniques is provided in Supplementary Table 1.

Despite these technical advances, it remains a challenge to functionally image large neuronal populations in 3D at single-cell resolution and on physiological time scales. Here, we demonstrate a two-photon light-sculpting technique capable of recording neuronal activity in a $500 \times 500 \mu\text{m}$ plane at high temporal resolution (~ 160 Hz) up to depths of $\sim 500 \mu\text{m}$ in awake, behaving mice with near single-neuron resolution ($5 \times 5 \times 10 \mu\text{m}$). By combining this advance with axial scanning, we can record neuronal calcium dynamics from hundreds of neurons in two planes (each $500 \times 500 \mu\text{m}$) at 10 Hz, or thousands of neurons at 3 Hz in a $500 \times 500 \times 500 \mu\text{m}$ volume, over an anatomical region that represents a major part of a cortical column²⁶.

Light sculpting based on temporal focusing (TeFo)^{27–30} is an approach that allows an effective decoupling of the axial and lateral confinement of excitation. It has been previously used to control the confinement of light in 3D within biological samples, mainly in the context of optogenetics^{31–33} or wide-field microscopy^{29,34,35}. Here we have used TeFo to shape the 3D point-spread-function (PSF) of our microscope near-isotropically in order to match it to the typical size of neuronal cell bodies in the mouse cortex ($\sim 10\text{--}15 \mu\text{m}$)³⁶. An appropriately designed excitation scheme based on a fiber-based chirped pulse amplified laser system (FCPA) and synchronization of the laser pulses to the voxel acquisition was then used for efficient excitation and point-detection of the fluorescence from the sculpted PSF.

The innovation of our approach, which we term scanned temporal focusing (s-TeFo), is based on the synergetic combination of three key ideas; **(1)** to excite the minimally required number of voxels per unit volume necessary to resolve the structure of interest, **(2)** to ensure a near-isotropic 3D shape of these excitation voxels and **(3)** to maximize the obtainable fluorescence signal-to-noise from each voxel by synchronizing the laser pulse repetition to the voxel acquisition rate. These conditions increase the volume acquisition rate by eliminating the unnecessary oversampling in the lateral (x,y) plane as is the case in many applications of conventional 2PM just for obtaining the necessary axial localization of excitation while maximizing the obtainable fluorescence signal-to-noise for a given average excitation power. The enlarged sculpted PSF volume in combination with the one pulse per voxel excitation scheme allows for a higher total signal-to-noise ratio from each GCaMP-expressing neuron, compared to the sum of multiple smaller excitation spots on the same neuron at the same excitation power density. This is in essence because larger voxels lead to a higher total collected fluorescence from each voxel and also because at a lower total number of voxels a lower repetition rate can be used - while maintaining the one-pulse per pixel condition - from which the signal benefits non-linearly (Supplementary Note 1 and Supplementary Figure 1). Thus, for the same obtainable signal to noise ratio we can either reduce the laser pulse energy per unit area and time ($\text{J}/(\mu\text{m}^2 \text{ s})$) which in turn improves cell viability or the higher obtainable signal-to-noise ratio in case of s-TeFo can be used to reduce the voxel dwell time and thereby speed up the voxel acquisition rate.

To achieve sufficient excitation of the fluorophores within our enlarged, sculpted PSF with a single laser pulse also necessitated the development of a laser system that is able to deliver increased pulse energy compared to conventional Titanium-Sapphire lasers at comparable pulse durations, and which operates at the MHz-repetition rate needed for high speed

imaging (Methods, Supplementary Note 2 and Supplementary Figure 2). This requirement goes beyond what is obtainable from typical regenerative amplified laser systems. We therefore designed and built a FCPA laser capable of providing sufficiently short pulses (~ 180 fs) at ~ 500 nJ suitable for efficient two-photon excitation at ~ 4 MHz repetition rate. Using this laser, we demonstrate the capabilities of our new imaging system by performing unbiased *in-vivo* calcium imaging of large V-FOVs (up to $500 \times 500 \times 500 \mu\text{m}$) at multi-hertz (3–160 Hz) time resolution in the mouse neocortex. Using our approach, we were able to capture up to 4,000 neurons in the aforementioned volume, comprising a major part of the mouse cortical column, and to monitor the dynamics of up to 3,000 active neurons in our experiments. To further demonstrate the potential of our volumetric imaging approach for capturing the collective population dynamics of a large number of neurons in other brain areas, we also recorded from $\sim 2,000$ neurons in the mouse hippocampus.

RESULTS

A sculpted excitation PSF enables large volume, high-speed imaging with a high signal-to-noise ratio

In standard 2PM, the voxel size is on the order of the diffraction limit of the laser light employed for two-photon excitation, primarily to achieve good axial sectioning. For light of $\sim 1 \mu\text{m}$ wavelength and a 0.8 NA objective, the excitation volume is therefore typically $\sim 1 \mu\text{m}^3$ (see Methods). Such a small focal volume results in slow volumetric imaging speeds, as a given V-FOV (e.g. $500 \times 500 \times 500 \mu\text{m}$) is then composed of a relatively high number of voxels ($\sim 5 \times 10^8$), all of which have to be scanned, leading to low volume acquisition rates (typically < 0.01 Hz, at best < 0.1 Hz). Given that the average size of neuronal cell bodies in the mouse cortex is in the range of $10\text{--}15 \mu\text{m}^3$ ³⁶, we reasoned that such high spatial resolution is not necessary to address a wide-range of questions in systems neuroscience. Thus we could trade spatial resolution for larger V-FOVs, i.e. for recording neuronal signals from larger population sizes, and at higher volume speed, while still maintaining near single-cell resolution (Supplementary Note and Figure 1). For a Gaussian beam, however, fundamental constraints from optics do not allow for arbitrary shapes of the excitation PSF spot size, as the lateral and axial localization of the excitation light are intrinsically coupled. For example, when focusing a laser to a laterally $5 \mu\text{m}$ wide spot, it would extend axially over $\sim 70 \mu\text{m}$ FWHM, and thus would fail to provide sufficient optical sectioning. To address this issue we used light-sculpting based on temporal focusing and generated a sculpted PSF of $\sim 5 \times 5 \times 10 \mu\text{m}$ FWHM diameter (Fig. 1a, b).

Realization of scanned light sculpting microscopy

A schematic of our s-TeFo imaging approach is depicted in Fig. 1a along with a measurement of the corresponding 3D PSF (Fig. 1b), and the schematic of the setup (Fig. 1c). The enlarged 3D PSF has an about 40-fold increased excitation cross-section of $\sim 20 \mu\text{m}^2$ compared to the PSF in diffraction-limited scanning. This required a corresponding increase in pulse energy in order to maintain a comparable power density ($\text{J}/(\mu\text{m}^2 \text{ s})$), which in turn necessitated the development of an alternative femtosecond laser that can provide sufficient pulse energies at repetition rates equal to or higher than the image acquisition rate.

We designed and built an amplified laser system based on fiber-optical components (Fig. 1d and Methods). The main advantage of our laser design is that the pulse repetition rate (pulses/sec) of our fiber-based chirped pulse laser amplifier (FCPA) can be matched and synchronized (Fig. 1e) to the voxel acquisition rate (voxels/sec), which in our case is in the range of 4–5 MHz, using a fiber-coupled acousto-optic modulator (FC-AOM). This is done while maintaining sufficiently high pulse energies in this regime (>500 nJ at laser output) to generate the required fluorescence signal with only one pulse per voxel. Furthermore, our FCPA has the advantage of providing sufficiently short pulses (~180 fs), exhibits high pulse-to-pulse energy stability (R.M.S <1 %) and does not require manual alignment and adjustments, due to its inherent all-in-fiber design (Methods).

It is important to note that the resulting power densities <5.5mW/μm² (or <2.5nJ/(μm² s)) are not higher than in conventional 2PM. To ensure that the overall average laser power (<220mW) in our *in-vivo* imaging experiments would not lead to any detrimental heating of the brain, we monitored *in-situ* brain temperatures under various imaging conditions using a small temperature sensor. We found a moderate (2–4 °C) increase in steady-state temperature depending on the experiment (Fig. 1f, g), which offset the brain surface cooling due to the cranial glass window and water immersion (base-line ~32–34 °C). Furthermore, the observed temperature increase was linear with average power, in line with our numerical simulations and previous studies^{37–39}, and similar to what has been observed in conventional two-photon imaging approaches. To confirm that thermal stress and injury were not associated with this modest rise in brain temperature, we examined the microglial and astrocyte response to laser scans of different intensities and modalities by immunohistochemistry (Iba1 and GFAP, respectively). We found no increase in microglial or astrocyte immuno-reactivity for laser powers at or below 220 mW, which were the highest effective average laser powers used in our experiments (Supplementary Figure 3 and Methods). We note that in principle non-linear photodamage due to multi-photon absorption can also occur. However, reported damage thresholds for this process are higher^{40,41} than the focal intensities used in our method when accounting for scattering losses due to imaging depth, which is also confirmed by the lack of any observed immune-reactivity in the above experiments.

High-speed calcium imaging of neuronal population activity across cortical layers and hippocampus in awake mice

As a first demonstration of the capabilities of our s-TeFo imaging system, we performed two-photon calcium imaging in Layer 2/3 (200 μm depth), Layer 4 (360 μm depth) and Layer 5 (470 μm depth) of the posterior parietal cortex (PPC) of awake head-fixed mice expressing the GCaMP6m/f calcium sensors (Fig. 2 and Methods). The mice were placed on a home-built mount that includes a running disk with adjustable counterweights and a weight-bearing jacket. Both contributed to minimizing the effects of brain movements (Methods and Supplementary Figure 4). The optimized voxel acquisition and excitation scheme of our s-TeFo system allowed us to record the activity of neurons at each plane at 158 Hz (Fig. 2c–e).

We typically recorded between 150 and 200 neurons in our imaging field-of view (FOV, 500×500μm) of which 80–120 showed activity, depending on imaging depth and the animal's behavior. Despite the high frame rate and low pixel dwell time (~250 ns), we could obtain sufficient signal-to-noise to extract calcium traces from the data with high quality (Fig. 2c–e). The overall characteristics of our recordings (Fig. 2c–e and Supplementary Video 1 & 2) such as the rise and decay time as well as fluorescence changes ($\Delta F/F_0$) are consistent with previous demonstrations of 2P imaging of neuronal dynamics in the mouse cortex^{17,22,23,25}. Our demonstrated frame rate of ~160 Hz and field-of-view of 500×500 μm, inside scattering brain tissue, outperforms previously demonstrated, unbiased imaging systems based on scanning two-photon microscopy^{17,21,22,25} when our obtained FOV, speed and depth (down to 500 μm) are considered collectively.

To extract the calcium dynamics of individual neurons from our imaging data, we applied a statistical method based on principal- and independent-component analysis (PCA/ICA)⁴²(see Methods for details). Manual inspection based on spatial segmentation of our raw imaging data sets revealed that, on average, typically 60±20 % of all identified neurons are active during a ~5 min recording window. We also compared the results of our PCA/ICA analysis to other common segmentation and Ca²⁺-trace extraction schemes based on a simple standard-deviation projection²⁵ as well as non-negative matrix factorization (NMF)⁴³, which has been shown to be able to discriminate Ca²⁺-signals between spatially overlapping neurons (Supplementary Figure 5). We found that in by and large PCA/ICA and NMF performed equally well on our s-TeFo imaging data, with a high correlation ($R=0.90\pm0.04$) between individual calcium traces (Supplementary Figure 5d, e).

Next, we explored the performance of our s-TeFo system for dual-plane imaging. Owing to the flyback and settling time of our long-range objective piezo translation stage, our dual-plane acquisition rates were limited to ~10 Hz in this case. We imaged separate planes located in layer 2/3 at ~150 μm depth and in layer 4 at ~350 μm depth (Fig. 3a–c and Supplementary Video 3). Again, we were able to record Ca²⁺ traces from individual neurons with sufficient quality and high temporal resolution (Fig. 3d). Our near-simultaneous dual-plane imaging capability allowed us to investigate neuronal correlations across cortical layers (Fig. 3e). Using our scan-TeFo approach, we could identify small clusters of spontaneous activity in each plane consisting of about 5–15 neurons, some of which showed correlated activity with a similarly sized group of neurons located in other cortical layers (Fig. 3e). The ability to capture correlated activity across cortical layers represents a key feature of our technique.

To demonstrate the full potential of our s-TeFo method for fast and unbiased, three-dimensional imaging, we performed continuous volumetric Ca²⁺-imaging over a V-FOV of 500×500×500 μm in the PPC of awake behaving mice at a volume rate of 3 Hz (Fig. 4 and Supplementary Video 4). This volume covers a major portion of a cortical column, and includes layers I–V in the mouse PPC cortex⁴⁴ (Fig. 4b). Most individual Ca²⁺-traces show pronounced maximum fluorescence changes ($\Delta F/F_0$), exemplifying the quality and signal-to-noise of the recording (Fig. 4c). In our data sets we typically identified several thousand (~2000–4000) neurons within our recording volumes, out of which a large portion (~60 %) showed activity during a typical recording time duration of ~ 5 min (Fig. 4b, d). Despite the

large volume size and high temporal resolution, we did not observe any obvious structures in the correlation matrix between neuronal pairs within or across layers (Fig. 4e). However, analyzing the spatial distribution of correlated neurons (Fig. 4f) reveals that most highly correlated ($R > 0.5$) neurons are spatially close ($< 50 \mu\text{m}$). Further, despite the long duration of the continuous volumetric imaging, we did not observe any obvious decline of the average basal fluorescence level as would be the case for photo-bleaching (Fig. 4d top).

In order to demonstrate the broader application of our method, we also performed Ca^{2+} -imaging in other brain regions. To this end we imaged the dorsal hippocampal CA1 region in awake, behaving mice. To access this brain-region we performed cranial window surgery following cortical aspiration, as previously reported^{45,46} (Fig. 5a, b). As in the cortex, we were able to record Ca^{2+} -traces with high temporal resolution (158 Hz, Fig. 4b, c and Supplementary Video 5) and signal-to-noise ratio, in a $500 \times 500 \mu\text{m}$ FOV. We also demonstrate the utility of s-TeFo in the hippocampus for imaging a volumetric FOV of $500 \times 500 \times 200 \mu\text{m}$ at ~ 6 Hz at depths of 0–200 μm below the cranial window (Fig. 5d, e and Supplementary Video 6). We were able to resolve and track reliably $\sim 2,000$ neurons expressing the red calcium indicator (jRGECO1a)⁴⁷, of which $> 1,400$ were active during the recording. As in the cortex, we find that correlated ($R > 0.3$) neurons are spatially close (Fig. 5f).

DISCUSSION

In this work, we have demonstrated near simultaneous 3D *in-vivo* Ca^{2+} imaging over multiple cortical layers using the new method of scanned temporal focusing (s-TeFo). Our light sculpting approach utilizes an enlarged, near isotropic PSF, so that a large volume can be sampled with the minimally required number of excitation voxels while maintaining near single-cell resolution. At the same time, this approach maximizes the obtainable signal-to-noise. This new approach leads to an optimal trade-off between signal, imaging speed, resolution and V-FOV, and is well-suited for applications where a reduced spatial resolution is sufficient for the benefit of highly improved volumetric FOV and speed. Our s-TeFo approach enabled us to monitor the network dynamics of thousands of neurons that comprise several cortical layers and the hippocampal cell assemblies in the mouse. In the mammalian neocortex these advances provide the opportunity to gain new insights into the computational principles of information processing and experimentally test a wide range of theoretical models of cortical computation^{48–50}.

We expect that future developments in laser technology will allow for more efficient excitation of molecular Ca^{2+} reporters which will be synergetic to the currently ongoing developments in making more efficient, red shifted fluorophores and Ca^{2+} indicators. On the molecular side, we foresee that the use of nuclear-localized and somatic-confined variants of Ca^{2+} indicators could provide further opportunities to obtain better single-cell resolution signals by eliminating signals originating from the neuropil. Finally, further increase in both the size of the V-FOV and the imaging speed are conceivable by employing faster scanners and combining our technique with approaches for parallelized excitation and detection such as spatial or temporal multiplexing^{21–23,25,51}. These strategies would be complementary to, and compatible with, our approach.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

METHODS

Fast two-photon volumetric imaging setup

The microscope setup is illustrated in Fig. 1c. It is built around a Sutter MOM scope (MP-285) and uses a galvo/resonant scanning mirror pair (6215H and CRS 12 kHz, Cambridge Technology). The optical and mechanical designs of the system were optimized for a low-magnification, high-NA objective (16x 0.8NA, Nikon). The scan and relay lenses were chosen such that the scanned TeFo spot on the grating is reimaged into the sample exciting a volume of $\sim 5 \times 5 \times 10 \mu\text{m}$ FWHM in the x, y and z direction, respectively. The objective was mounted on a long travel range, high-speed piezo stage (NPoint NPFocus1000, travel range 1mm), which allowed continuous volumetric image acquisition by a saw-tooth axial z-scanning of the objective. The piezo utilizes a high-speed driver (LC-402), which has an optimized flyback and settling time of $\sim 30\text{ms}$ over a $500\mu\text{m}$ travel range. Signal was collected using wide-angle optics and detection was performed using photo-multiplier tubes (PMTs) (H10770-40, Hamamatsu) in two color channels separated by a dichroic mirror (Semrock 525/50 and 624/40 with dichroic HC BS R561) protected by NIR blocking filters (Semrock HC 770/SP). Each PMT signal was amplified (14MHz bandwidth, 10^5 gain, Femto DHCPA-200) before it was recorded by a 120 MHz field-programmable gate-array (FPGA). The FPGA also allowed for synchronization of the voxel acquisition to the pulse repetition rate of the laser and to realize a scheme in which each voxel was excited only by a single laser pulse. This arrangement had the additional advantage that it allowed optimizing the sampling window with regard to the GCaMP fluorescence time response. By utilizing a high-bandwidth PMT amplifier (14 MHz) with a frequency higher than the repetition rate of the laser (4.1 MHz), we were able to trigger the FPGA digitizer board by individual laser pulses and thereby only collect the signal from the PMT only at its peak value. The laser intensity was modulated during the turnaround of the resonant scanner and with increasing image depth by a Pockels cell (Conoptics EO350-160-BK, driver 302RM). The temporal focusing stage consisted of a grating (1200l/mm, blaze wavelength 1080nm, Richardson 53009BK02-530R) and a temporal focusing tube lens (2", f=500mm). The optical design of the temporal focusing stage was based on 2" optics in order to ensure no transmission losses due to clipping and the large scan angles required to cover the $500 \times 500 \mu\text{m}$ FOV. The scan lens (f=110mm) and subsequent telescope (3.33:1, f1=60mm and f2=200mm, 3") resulted in a spot size of $200\mu\text{m}$ on the grating, which was translated to a temporally focused, lateral spot size of $\sim 5\mu\text{m}$ in the sample ($M_{\text{eff}} = 40$). Due to the relative angle between the incident light and the first diffraction order of the grating, the focal plane in the sample is slightly tilted along y with respect to the optical axis ($\sim 20\mu\text{m}$ over the $500\mu\text{m}$ FOV). The effective axial spacing between planes for the volumetric imaging experiments over $500\mu\text{m}$ was $\sim 12\mu\text{m}$ (43 planes – Fig. 4), and $10\mu\text{m}$ otherwise (hippocampus imaging – Fig. 5).

For image data acquisition, we used a high-speed 120MHz FPGA board (PXIe-7961R + NI 5734 Digitizer, National Instruments) with four input channels and the option to replace the internal time base by an external clock. This external clock signal (58MHz) came from the Yb-oscillator of the FCPA, as the minimum required frequency for this FPGA operation required >50MHz signals. In order to trigger data acquisition, the sync signal (4.1MHz) of the output of the FCPA was amplified and fed into one channel of the FPGA. We then maximized the fluorescence signal by shifting the relative time delay of the laser sync signal in respect to the external clock inside the FPGA. The control software was implemented in MATLAB, based on open-source software (Scanimage 5.1⁵²), but with custom developed code to allow for the above described, external synchronization to the laser pulse in the FPGA described above, and for one pulse per voxel image acquisition, as well as the incorporation of piezo settling times into volumetric sawtooth z-scanning. The acquisition settings for imaging were: temporal fill fraction 0.71, 2D image size: 120×120pixels, objective piezo axial flyback: 30ms.

Depending on depth, imaging was performed using a maximum of 220mW of time-averaged, i.e. effective, laser power that includes the effect of laser blanking (during scan mirror turnaround and flyback), out of the front lens of the objective (pulse energies < 50nJ). Taking into account the increased focal cross-section (20μm²) of the TeFo spot, this equates to a power density of <5.5mW/μm² (or <2.5nJ/(μm² s)). This is similar to or below the power density of standard, diffraction-limited two-photon microscopes, which typically utilize powers of up to several tens of mW within a focal cross-section of ~0.5 μm² (focal diameter of 0.8μm for our 16x, NA 0.8 objective). In our case the larger excitation volume allows to increase the imaging power compared to what is typically used for diffraction limited scanning. At our typical imaging parameters (500×500μm at 160 fps, or 500×500×500μm at 3 volumes per second) we used up to 220 mW. At these power levels we did not observe any increased brain heating compared to what has been observed with conventional 2PM microscopy (Fig. 1e, f) or any lasting photo-damage as reported by immunohistological markers (Supplementary Figure 3).

Design and construction of FCPA

For the experiments we used a custom-built Yb-fiber chirped pulse amplifier (FCPA - see Fig. 1d and Supplementary Figure 2). This laser produces 500nJ, 180 fs pulses with a central wavelength of 1035 nm and a repetition rate of 4.16MHz. In detail, the laser consists of an all-normal dispersion Yb-fiber oscillator that seeds an all-fiber integrated two-stage amplifier. The oscillator delivers positively chirped (pulse duration ~4 ps) 1-nJ pulses with a bandwidth of 20 nm centered around 1035 nm at a repetition rate of 58 MHz. In order to ensure high pulse fidelity, the oscillator is built using a 2-port scheme^{53,54}, where the output from the nonlinear polarization evolution ejection port is used solely for monitoring the mode-locked operation and generation of the electrical clock signal, and 50 percent of the 1-nJ output from the second output port is used to seed the amplifier. The already positively chirped pulses are further stretched in 10 m of single mode fiber (SMF) and 100 m of dispersion compensating fiber (DCF, provided by OFS Denmark) to a duration of about 300 ps^{55,56}. Since the stretcher fiber is not polarization maintaining (PM), a fiber polarization beam-splitter with non-PM SMF input fiber and PM-SMF output fibers, preceded by a fiber

polarization controller is used to ensure a stable polarization state and seed energy entering into the amplifier. Before the amplifier, a fiber-coupled acousto-optic modulator (FC-AOM; Gooch & Housego model 15200-.2-1.06-GaP-FO-GH-CAP) is used to reduce the repetition rate by a factor of 14, to 4.16 MHz (other integer reduction factors are also possible). In order to prevent detrimental effects caused by light propagating backwards through the system (such as amplified spontaneous emission or accidental back-reflections to the system), a high-power fiber-coupled Faraday isolator (AFW model PISO-30-L-7-0-3W-FB) is inserted after the FC-AOM, before the first amplification stage, which consists of a PM large mode area (LMA) 1+1×1 pump-signal combiner (AOFR model LC2010520HP) and a double-clad PM LMA Yb-doped fiber with a core diameter of 10µm (PLMA-YDF-10/125 from Nufern). After this first amplifier stage, a second fiber-coupled high-power Faraday isolator (AFW model PISO-30-L-7-0-5W-FB) is used before the light enters into the final amplification stage, consisting of a 6+1×1 pump-signal combiner (AOFR model LC7013229HP) with PM SMF signal input fiber and PM LMA output fiber, and a double-clad PM LMA Yb-doped fiber with 30 µm core diameter (PLMA-YDF-30/250-HI-8 from Nufern). The pump signal combiner ensures that only the fundamental mode is launched into the PM LMA fiber, and the splice of the combiner's output fiber and the active fiber is optimized to avoid coupling any light into higher-order modes. At the end of the active fiber, we spliced a 12-degree angle-polished end-cap, to prevent optical damage of the fiber end. After collimation of the output beam, a free-space high-power Faraday isolator (EOT model 08-I-1064-000-045) with low loss is used to prevent back-reflections from traveling back into the fiber amplifier. The pulses are then compressed to almost a Fourier limited duration (180 fs) using a pair of 1480 lines/mm gratings with high (~99%) diffraction efficiency, resulting in a >95% throughput of the compressor. The temporal profile and duration of the compressed pulses were optimized by fine-tuning the grating incidence angle and the distance between the two gratings, which controls both the ratio of the second to third dispersion orders and the amount of negative second order dispersion introduced by the compressor. The output pulses were characterized using second-harmonic frequency resolved optical gating (SH-FROG). For the imaging experiments, fine-tuning of the dispersion compensation was done so as to maximize the fluorescence yield of the GCAMP signal. Using a fast photodiode and a digital oscilloscope, the pulse-to-pulse energy stability was measured to be better than 1% RMS. A more detailed sketch of the laser design can be found in Supplementary Figure 2.

Image processing and data analysis

Image analysis was performed using custom software implemented in Matlab (Mathworks). First, images were corrected for the time-varying phase between the resonant mirror scan rate and laser repetition rate. This was done by having the FPGA record the relative time between the resonant mirror turnaround and the next laser pulse for every second image line (bidirectional scanning). This offset was stored in the image data, which allowed line-wise, sub-pixel image shifting in post-processing. Calcium signals were analyzed using a plane-wise PCA/ICA method based on Ref. ⁴². Spatial constraints imposed on the ICA segmentation such as size and shape criteria accounting for the somatic morphology reduced the likelihood that non-somatic signals, such as bright nearby axons or dendrites, would contribute to the identified spatial filters. This approach had the advantage of

requiring no *a-priori* knowledge about the location of neurons and, due to its automatic cell segmentation, is free of human bias. We typically kept 60 PCA components for analysis and the ICA-based segmentation was restricted to spatial filters that corresponded to the expected size (4–16 pixels) and morphology of neuronal cells, thereby minimizing possible contributions of neuropil and nearby dendrites or axons to the calcium signal. Calcium traces were extracted from the raw, unfiltered image data and F/F_0 was calculated as $F/F_0 = (F(t) - F_0)/F_0$, with F_0 being the median fluorescence intensity of each trace. For the high-speed F/F traces in Fig. 2c–e and Fig. 5c, a moving average of time width ~70 ms was applied prior to the signal extraction to provide a more reliable basis for the PCA/ICA analysis. For 3D imaging data, in order to account for neurons detected twice across adjacent planes, one out of two ICA filters was removed if they were spatially close (<5 voxels in XY), in subsequent planes and exhibited a pair-wise correlation (R) above 0.9. The custom-written codes for both hardware control and data analysis are available from the authors upon request.

Animal surgery and *in-vivo* calcium imaging of awake mice

Surgery and experimental procedures fulfilled the Austrian and European regulations for animal experiments (Austrian §26 Tierversuchsgesetz 2012 – TVG 2012) and were approved by the IACUC of The Rockefeller University. Adult (P90+) male and female C57Bl/6J wild-type mice (n=7) were anesthetized with isoflurane (2–3% flow rate of 0.5–0.7 l/min) and placed in a stereotaxic frame (RWD Life Science Co., Ltd. China). After removing the scalp and clearing the skull of connective tissues, a custom-made lightweight metal head-bar was fixed onto the skull with cyanoacrylate adhesive (Krazy Glue) and covered with black dental cement (Ortho-Jet, Lang Dental, USA or Paladur, Heraeus Kulzer, GmbH, Germany). A circular craniotomy (3–5 mm diameter) was then performed above the imaging site (posterior parietal cortex, PPC, centered at ~2.5 mm caudal and ~1.8 mm lateral; primary motor cortex, M1, ~–2.5 mm anterior and ~1.5 mm lateral; dorsal hippocampus ~2.0–2.5 mm caudal and 1.4–1.8 mm lateral to Bregma). With the skull opened and the dura intact, the GECI-carrying virus AAV8:hSyn-GCaMP6m and AAV2:CMV-NES-jRGECO1a (Ref. ⁴⁷ - Addgene plasmid # 61563) for cytosolic GCaMP6m and jRGECO1a, respectively, was injected at 4–12 sites (25 nl each, at 10 nl/min; titre ~10¹² viral particles/ml) with a 400-μm spacing forming a grid near the center of the craniotomy, at a depth of 400–450 μm below dura for PPC and 1200 μm for hippocampus. After the injections, a glass cranial window consisting of a 3–5 mm diameter, #1 thickness (0.16 mm) coverslip was implanted in the craniotomy, flush with the skull surface, and sealed in place using tissue adhesive (Vetbond). The exposed skull surrounding the cranial window was then completely covered with black dental cement to build a small chamber for imaging with water-immersion objective. To access the dorsal hippocampus, a cranial window was implanted after cortical aspiration as previously reported ^{45,46}. To prevent post-surgical infections and post-surgical pain, the animal were supplied with water containing the antibiotic enrofloxacin (50 mg/Kg) and the pain killer carprofen (5 mg/Kg) for a period of ~7 days. After surgery, animals were returned to their home cages for 2–3 weeks for recovery and viral gene expression before subjecting to imaging experiments. Extreme care was taken to ensure that the dura experienced no damage or major bleeding before and after cranial window implantation. Mice with damaged dura or unclear windows were euthanized and not used for imaging experiments.

During imaging sessions, the animals were head-fixed using a customized mount complemented with a head bar holder and a mouse body stabilizer (body jacket; Fig. 2b) and could freely run on a disc (200mm diameter, Fig. 2b). This considerably reduced animal induced motion of the brain during imaging (Supplementary Figure 4). A ventilation mask was placed in front of the mouse nose to provide air puff mechanical stimulation to the mouse whiskers and face as well as to provide gas anesthesia on demand. Typical imaging session lasted continuously for 2–10min.

Brain temperature measurements and immunohisto chemistry

Changes in brain temperature resulting from laser-induced heating were measured using a thermistor (10K3MCD1, Thermistor Probe Measurement Specialties, Inc.) and a thermocouple probe (Physitemp, IT-24P) for calibration. Probes were surgically implanted at a depth of ~200–300µm under the cranial window and located ~500µm away from the imaging FOV during recordings in order to ensure no direct illumination by the laser (thermistor diameter: 500µm, thermocouple diameter: 220µm). Raw temperature measurements were corrected for the direct effect of scattered laser light on the sensor as well as for feedback heating of the surrounding tissue by a correction factor of 0.47. This factor was calculated from side-by-side temperature time series measurements using both types of temperature sensor in agarose (10% vol) and taking into account the previously measured properties of the thermocouple device³⁹. Brain heating simulations were performed using published code (see Ref.³⁸), but taking into account the difference in wavelength (1035nm) as well as FOV (500µm diameter). Effects of pulsed light (4MHz) and beam scanning (3–160Hz) were deemed negligible compared to the time scales associated with tissue heating (10–100 seconds) and were thus not included in the simulation. Potentially neuropathological effects caused by laser-induced heating during brain imaging were assessed by immunohistochemistry. Awake head-fixed mice (wild-type) were subject to various laser power levels and scanning modalities (2D and 3D s-TeFo as well as 2PM) for 20 continuous minutes (conditions identical those in Fig. 2,4,5). Mice were perfused 16 hours after scanning and coronal brain sections (20µm thickness) were collected from the imaging site center and surrounding area. Brain sections were immunolabelled for microglial cells and astrocytes using rabbit-anti-Iba1 (Wako Chemicals) and mouse-anti-GFAP (Sigma-Aldrich) antibodies, respectively. Anti-rabbit Alexa 488 (Abcam) and anti-mouse Alexa 594 (Abcam) secondary antibodies were used to visualize primary antibody labeling. Slides were mounted in VECTASHIELD containing DAPI and brain sections were imaged at 20x magnification using a Panoramic 250 Flash slide scanner (3DHistech). Images were analyzed using ImageJ. Mean fluorescence intensities of ~0.5×0.5mm regions-of-interest at the center of the heating site were measured and divided by the mean intensity of a control area.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic and principle of scanned temporal focusing imaging system

(a) Schematic of the s-TeFo imaging. A large field-of-view is raster-scanned using an enlarged sculpted PSF and a one-pulse-per voxel excitation-acquisition scheme. Volumetric image acquisition is achieved by translating the objective axially (along z-axis) via a high-speed long-range piezo. Scale bar is 100 μ m. (b) Measured axial confinement of the sculpted point-spread-function (PSF) of the laterally symmetric TeFo-spot using 0.5 μ m sized beads. Corresponding x and z profiles showing lateral and axial confinement of excitation, a.u., arbitrary units. Scale bar is 10 μ m. (c) Overview of the s-TeFo microscope. The main dichroic mirror and a mirror are mounted on a slider in order to switch between two-photon scanning and wide-field epi-fluorescent imaging mode. EOM, electro-optical modulator, BS, beam-splitter, PBS, polarizing beam-splitter. (d) Sketch of the laser design, showing the ytterbium (Yb) oscillator seed, and two subsequent amplification stages based on polarization-maintaining large mode area Yb-doped fibers (Yb-PLMA). Free-space and in-fiber Faraday isolator prevent back-reflections. Pulses with a central wavelength of 1035nm are compressed to near Fourier limited duration (180 fs) using a pair of gratings. FCAOM, fiber-coupled acousto-optic modulator (e) The data acquisition is synchronized with the

laser by utilizing the Yb-oscillator signal as the external clock of the FPGA with an additional synchronization signal supplied by the output of the laser (see **c** and Methods for details). τ represents an electronic delay while AND performs a coincidence measurement of the photo-multiplier tube (PMT) signals with the sync signals. **(f)** Steady-state temperature increase measured *in-situ* in the mouse brain during imaging, depending on the effective average laser power used. Dashed line indicates highest average laser power used in the experiments. Simulations were done following Ref. ³⁸. **(g)** Measured (dots) as well as simulated (line) average temperature change from start of imaging, and thus laser illumination, for various scanning conditions. The discrepancy between simulation and experiment are attributed to blood vessels which provide increased heat transport away from the imaging site. Error bars denote standard deviation.

Figure 2. High-speed single plane Ca^{2+} -imaging in mouse posterior parietal cortex at 158fps using s-TeFo

(a) Sketch of the adult mouse brain, indicating the injection and imaging sites. M1 primary motor cortex, PPC posterior parietal cortex. Scale bar is 2mm. (b) Photograph of the animal mounted below the imaging objective via a head bar holder on a homebuilt running disc. The animal is additionally supported by a stabilizer attached to a body jacket. (c) Top left: Time averaged intensity projection image at Layer 2/3 (200µm depth) in mouse PPC expressing GCAMP6m. Right: Calcium traces of 114 active neurons identified in this plane. Each row shows the time-series activity of an individual neuron. Color indicates percent fluorescence changes ($\Delta F/F_0$); scaling is indicated by the color bar on the right. Typical calcium transients

consisted of an estimated $(30 \pm 10) \times 10^3$ detected photons (~ 3 photons per digitized count). Bottom left: Zoom-into a single trace (dashed box) is shown. Grey: raw data, red: moving average of 10 time points. **(d)** Same as in **c**, but at Layer-4 (depth of $360 \mu\text{m}$) in the same animal. **(e)** Same as in **c**, but at Layer-5 (depth of $470 \mu\text{m}$) of a GCAMP6f expressing mouse. The time-averaged laser powers used during imaging for the three different depths at the sample during imaging were approx. 100, 156 and 220mW in **c**, **d** and **e**, respectively. Scale bar is $100 \mu\text{m}$ in **c**, **d**. Representative recordings out of ten data sets.

Figure 3. Fast dual-plane Ca²⁺-imaging in mouse posterior parietal cortex at 10Hz

(a) Recording of two axially separated planes was achieved by rapid z-scanning of the objective with a piezo (10 Hz), between two imaging planes at 150 μm and 350 μm below the dura, corresponding to layer 2/3 and layer 4 respectively. (b, c) Time averaged intensity projection images of the two planes in PPC expressing GCAMP6m. (d) Extracted, raw Ca²⁺-traces of a 100sec recording from the planes shown in (a-c), displaying fluorescence changes (ΔF/F₀) over time. Dual-plane frame rate was 10Hz. Color codes for cortical layer of the neuron in (a). (e) Matrix showing correlation coefficient (R) calculated from all the time series shown in d, with neurons grouped by agglomerative hierarchical clustering per plane (color bar on the right). The dashed boxes show small clusters of correlated neurons

within each plane which also exhibit a positive correlation across the two planes (arrows 1 and 2) as well as two clusters that do not share correlations across layers (arrow 3). The time-averaged laser powers used during imaging for the two different depths at the sample during imaging were 73 and 170mW, in **b** and **c** respectively. Scale bar is 100µm in **b, c**. Representative recording out of three data sets.

Figure 4. Fast volumetric imaging of Ca^{2+} -dynamics across cortical layers in mouse posterior parietal cortex

(a) Three dimensional volume view (rendering) of the time-averaged, raw fluorescence recorded from a $500 \times 500 \times 500 \mu\text{m}$ volume in the cortex (PPC) in an awake mouse. (b) Number of active (blue) and inactive (grey) GCAMP6m expressing neurons identified in each axial plane. Depth from brain surface and approximate cortical layer correspondence indicated on top. (c) Distribution of maximum fluorescence change ($\Delta F/F_0$) of all neurons within the acquisition period. (d) Time-series heat map of all active neurons (2826 from a total of 4306) during a ~330sec recording. Volume acquisition rate is 3Hz. Zoom-in of individual traces shows distinct calcium transients (right panel). Graph on top of the heat map shows average fluorescence level of all neurons during the recording (black line) as

well as s.e.m (grey shading), indicating negligible photo-bleaching. **(e)** Matrix showing correlation coefficient (R) calculated from all the time series shown in **d**, with neurons grouped by agglomerative hierarchical clustering (color bar on the right). **(f)** Distribution of all neuronal pair distances with high correlation ($R > 0.5$). Red line shows scaled envelope of all neuronal pair distances, plotted in the inset. Results show enhancement of highly correlated, spatially close neuron pairs compared to all neuron pairs. The time-averaged laser powers used during volumetric imaging were between 95 and 195mW, depending on depth, cycling between these values in sync with the objective piezo and blanked during resonant mirror turnaround and piezo flyback (see Methods). Representative recording out of five data sets.

Figure 5. Fast volumetric imaging of Ca^{2+} -dynamics in mouse hippocampus CA1
(a) Schematic drawing of the hippocampal window preparation, indicating corpus callosum (CC), and region of hippocampus proper *Cornu Ammonis* (CA1, CA3) as well as dentate gyrus (DG). The red box shows the approximate imaging volume. **(b)** Time averaged intensity projection image at 100µm depth in mouse hippocampus CA1. Scale bar is 100µm. **(c)** Representative Ca^{2+} -traces of individual neurons imaged at 158fps in a single plane. Grey: raw data, red: moving average of 10 time points. **(d)** 3D rendering of time-averaged imaging volume (500×500×200µm). **(e)** Time-series heat map of all 1416 active neurons from a total of 1994 identified neurons within the imaging volume during a ~260sec recording. Images were acquired at a volume rate of ~6Hz. **(f)** Distribution of all neuronal pair distances with elevated correlation ($R > 0.3$). Red line shows scaled envelope of all

neuronal pair distances, plotted in the inset. This shows enhancement of correlated, spatially close neuron pairs compared to all neuron pairs. Time-averaged laser powers in the sample during imaging were 100mW in **c** and between 86 and 120mW in **d, e** depending on depth. Calcium indicator was cytosolic jRGECO1a. Representative recording of three data sets.