Wide field-of-view, multi-region, two-photon imaging of neuronal activity in the mammalian brain

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Two-photon calcium imaging provides an optical readout of neuronal activity in populations of neurons with subcellular resolution. However, conventional two-photon imaging systems are limited in their field of view to ~1 mm², precluding the visualization of multiple cortical areas simultaneously. Here, we demonstrate a two-photon microscope with an expanded field of view (>9.5 mm²) for rapidly reconfigurable simultaneous scanning of widely separated populations of neurons. We custom designed and assembled an optimized scan engine, objective, and two independently positionable, temporally multiplexed excitation pathways. We used this new microscope to measure activity correlations between two cortical visual areas in mice during visual processing.

Ensemble neuronal activity is of key interest in system neuroscience to understand sensory coding, motor output, and cognitive function. Measuring neuronal activity in populations of neurons in vivo is technically challenging, due to the densely packed neuropil and sensitive neuroanatomy that is best probed with minimally invasive approaches. Two-photon1 population calcium imaging in vivo² supports dense optical sampling of neuronal activity deep in scattering tissue such as the mammalian neocortex, and unambiguous identification of recorded neurons, particularly when genetically encoded indicators are employed3,4. Two-photon population calcium imaging is used to measure single-cell-level stimulus selectivity in dense local ensembles of neurons5,6 and learning and task-related activity in awake mice7, including those navigating in a virtual reality environment8.

However, conventional two-photon imaging systems retain cellular resolution over only a restricted field of view (FOV) of ~1 mm². This limitation precludes the simultaneous examination of areas large enough to encompass multiple cortical areas. Neuronal activity that supports sensory coding and motor output is distributed across multiple areas and millimeters of neocortex even in mice9,10 (Fig. 1a), thus new instrumentation is needed to obtain a cellular-resolution view into ongoing neural activity across extended cortical networks, which is essential for elucidating principles of neural coding.

Here we present a two-photon imaging system that preserves individual neuron resolution across a wide FOV (>9.5 mm²) and can simultaneously scan neuronal populations over extended cortical networks. The system uses custom optics (objective and scan engine) and dynamically and independently repositionable temporally multiplexed imaging beams. We refer to the system as a Trepan2p (Twin Region, Panoramic 2-photon) microscope. We demonstrate the utility and flexibility of this system with ultra-wide FOV imaging, arbitrary line scan imaging, dual region imaging offset in X/Y and/or Z, quad area imaging, and imaging with resonant scanning. As an example application, we used this new imaging system to measure activity correlations between neurons in different cortical areas in awake mice.

In the design of the Trepan2p imaging system, two criteria needed to be met: (1) optical access to an area >9 mm², and (2) the ability to measure neuronal activity across cortical areas at rates relevant for neuroscience analysis (e.g., activity correlations). The large FOV of the imaging system provides optical access to neurons in multiple cortical areas (Fig. 1b) without the need to move the imaging system or the sample. However, the increase in space-bandwidth product (compared to conventional two-photon imaging systems) naturally increases the time required for raster scanning the large FOV. Temporal multiplexing is the fastest method to simultaneously acquire multiple signals, but prior implementations have been fixed and not easily repositionable during an imaging session, or did not offer completely arbitrary beam positioning11–13. Therefore, we designed a new optical scheme for temporal multiplexing in which two beams are fully independently repositionable (Fig. 1c). These beams can be directed toward any region and depth within the large FOV to simultaneously scan and sample neuronal activity at time resolutions relevant for behavior and neural coding.

The imaging pathway is as follows (Fig. 1d). The light from an 80 MHz laser (Mai Tai, Coherent) is first split into two beams through polarization optics. One path (pathway 2) is delayed 6.25 ns relative to the other path (pathway 1). In this way the two pathways each have 80 MHz pulse trains delayed relative to one another by one-half of the period (Supplementary Fig. 1a). Custom-motorized steering mirrors (SM1 and SM2) impart independent solid angle deflections (Ω1 or Ω2)
Figure 1 Trepan2p system layout. (a) In the mouse, the primary visual cortex (V1) is surrounded by higher visual areas (HVAs; PM, posteromedial; AM, anteromedial; A, anterior; RL, rostralateral; AL, anterolateral; LM, lateralomedial; LI, laterointermediate), which are distributed across several millimeters of cortex (M1, M2, primary and secondary motor cortex, respectively; S1, S2, primary and secondary somatosensory cortex, respectively; Au, auditory cortex). A wide field of view (FOV) is required to image neuronal activity in these distributed cortical areas simultaneously. (b) The 3.5 mm FOV can encompass V1 and HVAs. (c) The individual imaging regions can be independently positioned and repositioned anywhere within the full FOV by the steering mirrors (SM1, SM2 in d) for XY position, and the tunable lenses (ETL in d) for independent Z positioning. (d) Two imaging beams are temporally multiplexed and independently positioned in XY and Z before the scan mirrors (SM-X, SM-Y). First, overall power is attenuated using a half-wave plate (λ/2), a polarizing beam splitting cube (PBS) and a beam block (BB). After a second λ/2 (used to determine the power ratio sent to the two pathways) and a beam expander (BE), a second PBS divides the beam into two pathways. Pathway 1 (in blue, p-polarization, indicated by the arrows) passes directly to a motorized steering mirror (SM1) for positioning in XY. Pathway 2 (in orange, s-polarization, indicated by the circles) passes to a delay arm where it travels 1.87 m further than pathway 1 using mirrors (M), thus delaying it by 6.25 ns before being directed to SM2. Directly before SM1 and SM2 are electrically tunable lenses (ETL) that can adjust the Z position (focal plane) of the pathways independently. The two pathways are recombined (beam recombination relay), and sent to X and Y galvanometer scanners (GS) that are connected by an afocal relay (expanded view inset). A scan lens (SL) and tube lens (TL) focuses the two multiplexed beams onto the back aperture of the objective (Obj). Fluorescence is directed to a photomultiplier tube (PMT) via an infrared-passing dichroic mirror (DM) and two collection lenses (CL1, CL2). for the two beams and are located in conjugate planes to the scan mirrors using afocal relays. Adjustments to the focal plane for each pathway are made using an electronically tunable lens (ETL; Optotune)\(^1\). Thus, each path is independently positionable in X, Y, and Z during imaging without moving the microscope or the preparation. Single photon PMT pulses are demultiplexed using a synchronization signal from the laser module, assigning detected photons to pixels in the associated imaging pathway (1 or 2) (Supplementary Fig. 1b,c) with minimal cross-talk between pathways (Online Methods and Supplementary Fig. 2).

Commercial microscope objectives and scan engines often used in two-photon imaging were insufficient for this application because they are designed for small scan angles and beam diameters, which ultimately limit the FOV over which individual neurons can be resolved in vivo to \( \sim 1 \) mm\(^2\) (larger FOV two-photon imaging has been limited to lower resolution applications such as blood flow imaging\(^2\)). Commercial macroscope objectives offer large FOVs for one-photon imaging, but they are not optimized for multiphoton excitation. Therefore, to provide high-resolution imaging over a larger FOV, both a new scan engine and a new objective had to be designed in concert, along with optics for rapidly reconfiguring the multiplexed imaging pathways.

Multi-element optical subsystems (afocal relays, scan lens, tube lens, and objective) were designed (Supplementary Figs. 3–6) to minimize aberrations at the excitation wavelength (910 ± 10 nm) across scan angles up to \( \pm 4 \) degrees at the objective back aperture (Supplementary Fig. 7a). In designing the custom optics, we prioritized the uniform performance (root mean squared (RMS) wavefront error, Supplementary Fig. 7) over the entire designed scanning range. In this way we could preserve cellular resolution anywhere within the large FOV. Subsystems can be diffraction limited on their own, but demonstrate additive aberrations when used together in a full imaging system. Optimizing the system as a whole (including all relays, scan lens, tube lens, and the objective), rather than optimizing components individually, ensured we would meet the desired performance. Because the imaging system would be used in volumetric imaging applications, small variations of the focal plane across the large FOV (field curvature) were allowed (Supplementary Fig. 7b). Additionally, we relaxed requirements for F-theta distortion (Supplementary Fig. 7c). Together, these strategies facilitated the design process.

We evaluated the experimental resolution of the Trepan2p microscope by measuring the excitation point spread function (PSF\(_{ex}\)) as the full width at half-maximum (FWHM) of the intensity profile of 0.2 μm beads (Fig. 2a–c). Radial FWHM was \( \sim 1.2 \pm 0.1 \) μm (mean ± s.d.) both at the center and at the edges of the FOV. The axial FWHM was 12.1 ± 0.3 μm at the center, and 11.8 ± 0.4 μm at the edges of the FOV (both measurements are mean ± s.d.; Fig. 2d). Because the custom objective is air immersion, changes in imaging depth (from the designed imaging depth) will introduce additional spherical aberrations. However, this is largely minimized due to the
moderate numerical aperture (NA) and thus the PSF_{ex} shows only minor changes as a function of imaging depth (Fig. 2d). The use of the tunable lens for focal plane alterations can affect the PSF_{ex}^{14}, though in the range it was typically used (±50 µm) it has a small impact on the PSF_{ex} (Supplementary Table 1).

The Trepan2p system enables structural and functional imaging of neuronal resolution in vivo. To locate the primary visual cortex (V1) and higher visual areas (HVAs) in the mouse10, we used intrinsic signal optical imaging17 (Supplementary Fig. 8 and Supplementary Video 2). To demonstrate the functionality of the Trepan2p system, we performed two sets of in vivo imaging in transgenic mice that expressed the genetically encoded calcium indicator, GCaMP6s3, in neocortical pyramidal neurons (Emx1-ires-Cre x Rcl-GCaMP6s/A96). In the first set of experiments, we explored the optical access to neurons across the large FOV. To locate the primary visual cortex (V1) and higher visual areas (HVAs) in the mouse10, we used intrinsic signal optical imaging17 (Supplementary Fig. 8). We then imaged neuronal-activity-related GCaMP6s signals in a large cortical region (Fig. 3a–e, Supplementary Fig. 12 and Supplementary Video 3). The full large FOV was scanned with a single beam at ~0.1 frame/s (3.5 mm wide FOV; 2,048 × 2,048 pixels; 3 µs dwell time per pixel), and contained in it V1 and at least six HVAs. Undersampling and alternative scan...
strategies such as arbitrary line scanning (Supplementary Fig. 13) can increase the temporal resolution\(^{18,19}\), and stimulus presentation durations can facilitate the capture of stimulus-related events.

Neurons were readily detected throughout the entire FOV based on spiking activity reported by GCaMP6s (5,361 neurons detected; Fig. 3d,e). Thus the Trepan2p system provides cellular-resolution imaging of AM/PM neurons.

Figure 4 Temporally multiplexed, independently repositionable imaging pathways for simultaneous scanning two regions. (a) The individual imaging regions can be independently positioned and repositioned anywhere within the full FOV. (b) Within the same session, without moving the mouse or the microscope, the two pathways were moved to various configurations (left) to image neuronal activity (3.8 frames/s per region) (left, segmented active ROIs within the 500 \( \mu \)m imaging region; right, five example traces from each region). (c) There is no lower limit to the XYZ separation between imaging pathways (from the mechanical point of view). In this imaging session (9.5 frames/s per region), the XY locations were identical and the pathways only differed in the Z depth (left, segmented active ROIs within the 500 \( \mu \)m imaging region; right, five example traces from each region). (d,e) By combining temporal multiplexing (pathways 1 and 2) with changing the offset voltage serially on the galvanometer scanner, four regions can be rapidly imaged (ten frames/s per region). Pathways 1 and 2 are positioned at the same XY location and offset in Z. The galvanometers serially position the imaging region of each pathway anywhere within the larger field of view (left, segmented active ROIs within the 400 \( \mu \)m imaging region; right, five example traces from each region) (e). (f) Resonant scanning was performed for faster frame rates (30 frames/s per region) (left, segmented active ROIs within the 500 \( \mu \)m imaging region; right, six example traces from each region; Supplementary Video 7). (g) Neuronal activity was imaged in two regions (20 frames/s per region), V1 and an ROI encompassing retinotopically matched regions of AM and PM, simultaneously. Visual stimuli, either drifting gratings or a naturalistic movie, were used to evoke responses (left, segmented active ROIs within the 400 \( \mu \)m imaging region; right, five example traces from each region and for each visual stimulus). (h) Ca\(^{2+}\) signals were used to infer spike times and examine correlations. Activity correlations were measured between pairs of cells, each pair consisting of a V1 neuron and a neuron in AM or PM. These correlations were higher during presentation of the naturalistic movie compared to those during the drifting gratings (cross-correlation with gratings, mean \( \pm \) s.e.m.: 0.0157 \( \pm \) 0.0003; with naturalistic movie: 0.0218 \( \pm \) 0.0003; \( N = 12,160 \) neuron pairs; \( P < 10^{-11} \), rank-sum test). The neurons on both axes were ordered from low to high average correlation for presentation clarity on the left (naturalistic movie), and the same ordering is used on the right (gratings). Imaging depth for both pathways was 235 \( \mu \)m. For all panels the vertical scale bar is 200% AFF and the horizontal scale bar is 10 s. All imaging depths are indicated in the panels. The depth offset in pathway 2 (orange) was accomplished using the tunable lens.
optical access across the entire FOV (9.6 mm²), which encompasses more than six different mouse cortical areas within a single FOV.

For many neuroscience experiments, the activity dynamics of interest are restricted to two or more subregions of the large FOV. To capture neuronal activity correlations, these regions should be imaged simultaneously. The fastest approach to simultaneously image multiple regions is temporal multiplexing, as implemented here. To demonstrate the flexibility of this approach, we performed experiments with a variety of configurations of the twin imaging pathways (Fig. 4a and Supplementary Video 4) in animals expressing GCaMP6s in neocortical pyramidal neurons (TITL-GCaMP6s x Emx1-ires-Cre x Rosa26-Lnlt(1TA)). While presenting visual stimuli to the mouse (a naturalistic movie), we simultaneously recorded activity in several different pairs of cortical areas, and at different depths (512 × 512 pixels, 3.8 frames/s per region, Fig. 4b). The imaging regions can be placed arbitrarily close to each other. To demonstrate this, we placed the two imaging paths at the same XY location, but offset their positions in Z (512 × 256 pixels, 9.5 frames/s per region, Fig. 4c and Supplementary Video 5). Next we explored quad region imaging. Each of the dual imaging pathways were configured to alternate (every other frame) between two different regions. Thus, we imaged four different XY regions, each at 10 frames/s per region (250 × 100 pixels, Fig. 4d; 512 × 512 pixels, 1.9 frames/s per region, Supplementary Video 6). We also imaged four regions that differed in the XY locations and Z (250 × 100 pixels, 10 frames/s per region, Fig. 4e). Resonant scanners provide a way to increase the frame rate in raster scanning modes, and this is completely compatible with the optical system we present here. We used a resonant scanner to achieve 30 frames/s imaging in two regions simultaneously (512 × 256 pixels, 30 frames/s per region, Fig. 4f and Supplementary Video 7).

As an example experiment, we imaged neuronal activity in different cortical areas simultaneously, V1 and in medial HVAs AM and PM (250 × 100 pixels, 20 frames/s, Fig. 4g), while presenting two different visual stimulus videos (a naturalistic movie or drifting gratings). Pairwise correlations between cortical areas were calculated from spike time courses inferred from the GCaMP6s signals. This analysis revealed an increase in correlated activity between neurons in V1 and neurons in areas AM and PM when the naturalistic movie is presented, compared to gratings (cross-correlation with gratings: mean ± s.e.m.: 0.0157 ± 0.0003; with naturalistic movie: 0.0218 ± 0.0003; N = 12,160 neuron pairs; P < 10⁻¹⁰; rank-sum test; Fig. 4h). Such cross-area correlation analysis between densely sampled populations of neurons was made possible by both the large FOV and temporally multiplexed scanning. These imaging regions can be as large or small as needed for the experiment and temporal resolution (e.g., a 2.5 mm FOV at two different z-depths; 2,048 × 1,024 pixels, 0.8 frames/s; Supplementary Fig. 14 and Supplementary Video 8), and two regions can be set to scan complementary halves of the FOV (as in ref. 11). Thus, the repositionable temporally multiplexed imaging beams can be reconfigured within an experiment for a variety of measurements.

In this work we have addressed a major barrier to progress in two-photon imaging of neuronal activity: the limited FOV. Using novel optical systems, the Trepan2p has expanded optical access to neurons in an area greater than 9.5 mm², using mostly cost-effective COTS components. This large FOV can contain, in mice, primary visual cortex and six higher visual areas. Rapidly repositionable multiplexed imaging paths make it possible to capture neural activity in two arbitrarily selected subregions simultaneously within the large FOV.

The results presented in this manuscript demonstrate the utility of the Trepan2p system. The large working distance (8 mm) of the air-immersion objective can facilitate integration with head-fixed behavior experiments. Moreover, in principle the same instrumentation can be applied to image neural activity in many animal models whose cortical organization extends beyond the spatial limits of conventional two-photon microscopy, such as multiple orientation columns in the primary visual cortex of cats or non-human primates. The methodological approach and technologies presented here provide a flexible platform for measuring neural activity correlations and dynamics across extended cortical circuitry with individual neuron resolution. Beyond measuring neural activity, the Trepan2p can be applied for other physiological measurements such as blood flow and structural imaging in cleared tissue.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.L.S. conceived the Trepan2p imaging system. J.N.S. and S.L.S. designed and engineered the system. J.N.S. developed the demultiplexing electronics, designed and optimized the optical systems, wrote the software, and constructed the system. M.W.K. consulted on optical optimizations. J.N.S., I.T.S., and S.L.S. performed the animal experiments. J.N.S. and S.L.S. analyzed and interpreted the data. J.N.S. and S.L.S. wrote the manuscript with input from all authors. I.T.S. and S.L.S. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Optical design and simulations. Relay, scan, tube, and objective lens systems were modeled in OpticStudio (Zemax, LLC). Three optical subsystems ((1) relay, (2) scan and tube lens, (3) objective) were first designed, modeled, and optimized individually. After initial isolated design, the system was simulated and optimized as a whole to ensure there would be no unforeseen additive aberrations between subsystems. One of the main difficulties in designing a highly corrected optical system is dealing with chromatic aberrations. We decided to focus our design around a narrow wavelength range suitable for GCaMP excitation (910 ± 10 nm). This eased our requirements for correction of chromatic aberrations. For the relay and scan and tube systems, we used all commercial off-the-shelf (COTS) lenses (Thorlabs and OptoSigma) to minimize cost (Supplementary Figs. 3–5). These lenses were coated with an infrared anti-reflective coating that had <0.5% reflectance at 910 nm.

To obtain diffraction limited performance (Supplementary Fig. 7a) in the simulated design and sufficient performance with in-house assembly, the cost-effective COTS components were supplemented with two custom-cemented doublets in the objective (Supplementary Fig. 6). The custom-cemented doublets were manufactured by Rainbow Research Optics, Inc. (Centennial, CO) and were coated with a broad-band anti-reflective coating (<1% 400–1,100 nm). The other elements were COTS (Thorlabs and OptoSigma) and were coated with a broad-band anti-reflective coating (<1% 525–925 nm; OptoSigma). We relaxed the requirement to have the focal plane be flat, as slight deviations of the focal plane across the FOV would not affect our ability to capture neural dynamics. The resulting Trepan2p field curvature is <20 µm (Supplementary Fig. 7b). Additionally, the F-theta distortion is minor (<5%) (Supplementary Fig. 7c). After the individual subsystems were designed and optimized, the system was optimized as a whole, while further refinement of spacing was made to allow for optomechanical limitations (lens housing, scanning mirrors). Complete lens data of the Trepan2p system is given in Supplementary Figures 3–6.

Assembly. To assemble the lens subassemblies, we used Thorlabs slotted lens tube system (2 inch) and retaining rings. Axial separations between elements were measured using digital calipers along with a custom metrology system using a digital camera (Thorlabs, DCC1545M) and telecentric lens. All lenses were mounted and measured based on the plano or concave side to minimize tilt/tip and secured on the convex side with a retaining ring after alignment. Decentering of lens elements was done manually with aid of the camera measurement system. The smaller negative elements (surfaces 1,3,23,25,28,30,48,50,53,68; Supplementary Figs. 3–6) were mounted in the 2 inch lens tube system using a three-dimensional (3D) printed adaptor (Quickparts). Subassemblies were mounted on 60 mm cage system (Thorlabs). Galvonometer and resonant scanners were mounted on a XY translator (Thorlabs, CXY2), and this was attached to a 60 mm cage cube (Thorlabs, LC6W), which was in turn connected to the other optical subassemblies through the cage system. The XY translator allowed for precise positioning of the scanning mirrors and allowed for offset due to the thickness of the mirror.

Within the systems there are natural axial separations between subassemblies that can be used as compensators. As the system is assembled, locations of afocal space at conjugate planes (Supplementary Figs. 3–6) are checked for collimation as designed in the system (Thorlabs, SI100).

Tolerancing and sensitivity analysis. A key component in optical design and manufacturing is tolerance analysis21. A system may have high simulated performance, but if the required precision of optical alignment is too high, the system may not be easily manufactured or may be too costly. For rapid turnaround and to keep costs low, we sought to design and build the Trepan2p system in-house, and to use primarily COTS components. The RMS wavefront error of the designed system shows diffraction-limited performance across the field of view (Supplementary Fig. 7a). The predicted excitation PSF measurements are ~8.8 µm on axis, and ~8.9 µm at a radial distance of 1.75 mm (edge of the 3.5 mm field of view) (Supplementary Table 1). These excitation PSF measurements are the FWHM of the square of the illumination PSF (Huygens PSF) output of the Zemax simulation of the system (see Excitation PSF and individual neuron resolution).

To ensure adequate performance of the system, we performed tolerancing analysis and designed compensating features to adjust for residual aberrations after assembly. For the COTS elements used, we used the following tolerances: radius of curvature = 5 fringes, element thickness = 0.1 mm, tip/tilt (X and Y) = 0.05 degrees, S + A irregularity = 0.25, and index of refraction = 0.001. For the element-to-element tolerances we used the following: axial separation = 0.175 mm, decenter (X and Y) = 0.11 mm, and tip/tilt (X and Y) = 0.150 mm. Within the systems there are natural axial separations between subassemblies that can be used as compensators (red boxed spacing, Supplementary Figs. 3–6). Setting these axial separations to be compensators, Monte Carlo simulations of the system performance (tolerance analysis) was performed, using a merit function that measured RMS wavefront error across the FOV and penalized the system for vignetting. The nominal merit function value for the designed system is 0.043. We performed 2,500 cycles of Monte Carlo simulation for the system with only axial displacement compensators (blue distribution, Supplementary Fig. 7d), and found that the expected system performance would not be sufficient. To increase the system performance, we designed into the objective optomechanical assembly two additional compensators: the axial separation between surfaces 76 and 77 and the XY decenter of custom element 1 (Supplementary Fig. 6). This was accomplished by mounting custom element 1 in an XY translator (Thorlabs, CXY2) and by using an adjustable adapter attached to this translator (Thorlabs, SM2P). With these three additional degrees of freedom, the Monte Carlo simulated performance considerably improved (green distribution, Supplementary Fig. 7d). The measured performance (resolution) of the system as assembled (Fig. 2), best matches simulated systems with merit functions in the range of 0.14–0.15 (red bar, Supplementary Fig. 7d). The inclusion of designed compensators (especially those within the objective) were critical to the success of our in-house assembly strategy.

The inclusion of the compensators in the assembly process provides a way to approach ideal performance, yet they cannot completely compensate for all imprecisions in the optical system. Tolerances in element manufacturing and misalignments in assembly remain and will lead to deviations from simulated ideal performance. During sensitivity analysis on this system, with the inclusion of the compensators, we found that the worst offenders are the decenter of the elements within the objective assembly. Decentering of these elements leads to an increased level of coma aberration. During exploration of the Monte Carlo simulations (part of the tolerancing process), we observed the aberrations in the simulated wavefront. The most common aberrations were typically coma aberration with some level of spherical aberration. This is likely true in our assembled system leading to the differences between simulated and experimental excitation PSF.

Animals. All procedures involving living animals were carried out in accordance with the guidelines and regulations of the US Department of Health and Human Services and approved by the Institutional Animal Care and Use Committee at University of North Carolina. C57Bl/6 mice were housed under a reversed 12 h-light/12 h-dark light cycle with ad libitum access to food and water. Two lines of transgenic mice were used that express GCaMP6s in the striatum (Supplementary Figs. 2, 6). Ai96 (RCL-GCaMP6s), crossed with Em1-Cre mice (Jackson Labs stock #005628; Fig. 3) and (2) Cre/Tet-dependent GCaMP6s mice, Ai94 (TITL-GCaMP6s) triple crossed with Em1-Cre and ROSA:LN1:tTA mice (Jackson Labs stock #011008; Fig. 4)22. Ai94 and Ai96 lines were kindly provided by H. Zeng and the Allen Institute. We used a transgenic mouse in which a tyrosine hydroxylase (TH) promoter directs expression of Cre recombinase (TH-Cre; Jackson Labs stock #008601). In this mouse, the ventral tegmental area (VTA) was injected with adeno-associated virus particles (AAV5-DIO-ChR2(H134R)-EYFP; 500 nL bilateral at an infusion rate of 100 nL/min). The animal was euthanized 3 months after surgery. TH-expressing terminals arising from the VTA were imaged in the striatum (Supplementary Fig. 2). Additionally, a Thy1-GFP O-line mouse, which expresses GFP largely in Layer V, was used to generate Supplementary Video 2.

Surgery. Mice were deeply anesthetized using isoflurane (5% for induction, 1–2% for surgery) augmented with acepromazine (0–0.4 mg/kg body weight, i.p.). Physically activated heat packs (SpaceGel, Braintree Scientific) were used
to maintain the body temperature during surgery. The scalp overlaying the right visual cortex was removed, and a custom head-fixing imaging chamber\textsuperscript{23} with 5-mm diameter opening was mounted to the skull with cyanoacrylate-based glue (Oasis Medical) and dental acrylic (Lang Dental). A 4-mm diameter craniotomy was performed over visual cortex. Carprofen (4.4 mg/kg body weight, s.c.) was administered postoperatively to all mice that underwent recovery surgeries before returning them to the home cage. Mice were mounted on a holder via the chamber\textsuperscript{23}. For intrinsic signal optical imaging, this chamber was filled with a physiological saline containing (in mM): 150 NaCl, 2.5 KCl, 10 HEPES, 2 CaCl\textsubscript{2}, and 1 MgCl\textsubscript{2}.

**Intrinsic signal optical imaging (ISOI).** Custom instrumentation was adapted from the work of Kalatsky and Stryker\textsuperscript{17}. Briefly, two F-mount lenses with respective focal lengths of 135 and 50 mm (Nikon) formed a tandem lens macroscope, which was attached to Dalsa 1M30 CCD camera (Teledyne DALSA). This configuration provided a 4.7 mm × 4.7 mm field of view (21.2 mm\textsuperscript{2}). Acquired images were binned 2 × 2 spatially, resulting in a final pixel size of 9.2 μm × 9.2 μm. The pial vasculature was illuminated through a green filter (550 ± 50 nm, Edmund Optics) and the vasculature map was captured through a second green filter (560 ± 5 nm). From the pial surface, the macroscope was then focused down 600 μm where intrinsic signals were illuminated with halogen light (Asahi Spectra) delivered by light guides and focusing probes (Oriel) through a red filter (700 ± 38 nm, Chroma). Reflected light was captured through a second red filter (700 ± 5 nm, Edmund Optics) at the rate of 30 frames per second with custom-made image acquisition software (adapted by J.N.S. from code kindly provided by David Ferster, Northwestern University). Mice were head-fixed 20 cm from a flat 60 cm × 34 cm (width x height) monitor, which was tilted toward the mouse 17.5° from vertical with their head angled to their right to cover the visual field (110° by 75°) of the contralateral eye. A light anesthetic plane was maintained with 0.5% isoflurane during imaging, augmented with acepromazine (1.5–3 mg/kg), and the body temperature was kept at 37 °C using feedback-controlled electric heat pad systems (custom-built).

**Visual stimuli.** A drifting white bar on a black background (elevation and azimuth direction; 3° thick) was used to map retinotopy during ISOI. These were produced and presented using MATLAB and the Psychophysics Toolbox\textsuperscript{24,25}. A corrective distortion was applied to compensate for the flatness of the monitor\textsuperscript{26} (code is available online, http://labrigger.com/blog/2012/03/06/mouse-visual-stim/). During calcium imaging, drifting gratings (0.04 cycles/°, 2 Hz, eight directions, 10 s/direction) or a naturalistic movie were displayed on a small video display located 6.3 cm from the left eye. The naturalistic movie was from a helmet-mounted camera during a mountain biking run. The stage was moved axially in 0.4 μm, 550 μm, (semi-axes of 1.5 μm, 0.4 μm, and 0.4 μm). Thus < 200 mW in the Trepan2p provides the same power density as 21.5 mW in a high NA imaging system. The larger excitation volume allows us to increase the imaging power without the damage seen in higher NA objectives\textsuperscript{27}. With typical imaging parameters (512 × 512 at 3.8 frames/s, 0.5 mm imaging region) up to 250 mW per channel could be used with no observed damage when imaging somas ~250 μm deep. Damage was observed with these parameters if the imaging plane was moved to the surface of the dura. When imaging superficially, the power was kept below 100 mW. Assessment of damage due to laser intensity was based on visual morphological changes to the appearance of the dura mater and/or continuously bright cell bodies. As a positive control, we used high (>300 mW at ~200 μm below the pial surface) continuous laser illumination, which caused photodamage evidenced by bright cell bodies and brighter-than-normal fluorescence of the dura (control mouse was not used for subsequent activity imaging). At the laser powers used for the imaging in this manuscript (<200 mW) such damage was not observed.

**Image analysis for neuronal calcium signals.** Ca\textsuperscript{2+} signals were analyzed using custom software in MATLAB (Mathworks). Neurons were identified and segmented using either a pixel-wise correlation map\textsuperscript{6} or a pixel-wise kurtosis map. For the correlation map, each imaging frame of the calcium imaging video was first Gaussian filtered in XY (sigma = 2 pixels), and then the kurtosis for the time series of each pixel location was computed using the filtered version of the video. This yields a single real-valued map with enhanced contrast for regions of interest that exhibit larger fluorescence transients. The maps were segmented into individual ROIs (neurons and processes) using a locally adaptive threshold, and ΔF/F traces were calculated from the raw, unfiltered data. An exponential moving average of time width ~150 ms was applied to the high speed ΔF/F traces in Figure 4d–h all other data was left unfiltered. The activity transients shown in Figure 3 are typically captured in only one frame owing to the acquisition frame rate. For the correlation analysis, spike inference\textsuperscript{20,28} was performed on the raw F/F traces before computing correlations. For pairwise correlations >0.5, traces were inspected for cross-talk. If the ROIs were present in the same XY positions in both imaging pathways and had high correlation values, the ROI with the weaker signal was determined to be due to cross-talk and was omitted (see Cross-talk below).

**Arbitrary line scan analysis.** Line scan data were arranged into a location-on-path versus time plot. The kurtosis of the data was taken over the acquisition frame rate. For the correlation analysis, spike inference\textsuperscript{20,28} was performed on the raw F/F traces before computing correlations. For pairwise correlations >0.5, traces were inspected for cross-talk. If the ROIs were present in the same XY positions in both imaging pathways and had high correlation values, the ROI with the weaker signal was determined to be due to cross-talk and was omitted (see Cross-talk below).

**Excitation point spread function measurements and simulations.** In two-photon imaging, the optical resolution of the system is evaluated by the excitation point spread function (PSF\textsubscript{ex}), which is the square of the illumination point spread function (IPSF\textsuperscript{2}) (ref. 29). To evaluate the PSF\textsubscript{ex}, submicrometer beads were imaged. Submicrometer fluorescent beads (0.2 μm, Invitrogen F-8811) were imbedded in a thick (~1.2 mm) 0.75% agarose gel. 50 μm z-stacks were acquired, each centered at one of three depths (55 μm, 275 μm, 550 μm). The stage was moved axially in 0.4 μm increments (Δz\textsubscript{stage}). At each focal plane five frames were acquired and averaged to yield a high signal-to-noise image. Due to the difference between the refractive index of the objective immersion medium (air) and the specimen medium (water), the actual focal position within the specimen was moved an amount Δz\textsubscript{focal} x Δz\textsubscript{stage} (ref. 30). The factor 1.38 was determined in Zeemax and slightly differs from the paraxial approximation of 1.33. These images were imported into ImageJ and the μm/pixel scaling for the XY and μm/frame (step) were input. For the axial PSF\textsubscript{ex} XZ and YZ images were created at the center of a bead, and a line plot was made at an angle maximizing the axiaispread, thereby preventing underestimation of the PSF\textsubscript{ex} due to tilted focal shifts. For the radial PSF\textsubscript{ex} an XY image was found at the maximum intensity position axially. A line scan in X and Y was made. Gaussian curves were fit to the...
individual line scans to extract FWHM measurements. The radial PSF$_{ex}$ values are an average of the X PSF$_{ex}$ and Y PSF$_{ex}$, and the axial PSF$_{ex}$ is an average of the axial PSF$_{ex}$ found from the XZ and YZ images. Excitation PSF measurements were performed both on axis and at the edges of the field of view for both imaging pathways. For measurements using the electronically tunable lens (Supplementary Table 1), the focal plane was first set at 275 μm using the XYZ stage with a current to the tunable lens of ~75 mA for zero offset. The focal plane was then shifted to either 225 μm or 325 μm (for ± 50 μm change in focal plane) by applying a previously calibrated current to the tunable lens. A Z-stack was then acquired as previously described. The minimal laser power for excitation was used to minimize the effects of nonlinear expansion of the apparent excitation PSF. Data reported (Fig. 2 and Supplementary Table 1) are the mean ± s.d. of eight beads.

For the simulated PSF measurements (Supplementary Table 1), at each of the three simulated depths (Surface 85 in Supplementary Fig. 6) the optimal focus (Surface 85 in Supplementary Fig. 6) was found (minimal RMS wavefront error). At this location and at simulated locations ± 25 μm from this location in 0.4 μm steps (this represents the steps of the stage as explained previously, Δstage), the Huygens illumination PSF (IPSF) was simulated. These images were imported into ImageJ and processed as previously described (accounting for the step size change: ΔIPSF = 1.38 x Δstage) to generate a Gaussian curve fit. This Gaussian was then squared to yield the excitation PSF (= IPSF$^2$). The FWHM of this squared Gaussian is reported as the simulated PSF$_{ex}$.

Trepan2p instrumentation. Laser pulses from a Ti:Sapphire laser ( Mai-Tai; Newport) with an automated pre-chirper unit (DeepSec; Newport) were attenuated using a half wave plate followed by a polarization beam splitting cube. Similar polarization optics were used to split the beam into two pathways and control the relative power between the two paths. Prior to splitting, the beam was expanded using a 3× beam expander (Thorlabs). One beam travels directly to a custom motorized steering mirror, and the other beam is first diverted to a delay arm, and subsequently to a separately controlled steering mirror (Fig. 1d). The delay arm is designed to impart a 6.25 ns temporal offset to the pulses in one beam (1.875 m additional path length). As the laser pulses are delivered at 12.5 ns intervals (80 MHz), they are evenly spaced in time at 160 MHz after the two beams are recombined (Supplementary Fig. 1a).

The steering mirror in each pathway imparts a solid angle (Ωx or Ωy) deflection to the beam before recombination (Fig. 1d). It is this angle that determines the central locations (X1, Y1; X2, Y2) of individual imaging subareas within the larger FOV. The two beams are combined and relayed to the X-axis galvanometer scanner using the third polarization cube located in the middle of an afocal pupil relay (Supplementary Fig. 3). The 2× polarization beam combining cube (Edmund Optics) was positioned in the afocal relay such that the focal point of the relay was outside the cube. This was to avoid high-intensity damage to the cemented surface within the cube. A similar afocal pupil relay (Supplementary Fig. 4) is present between the X-axis and Y-axis galvanometers (Cambridge Technologies). In this manner the two beams are simultaneously raster scanned (field size determined by scan amplitude), but with independently controlled spatial positions (determined by Ωx, Ωy). Immediately before the steering mirror in pathways 1 and 2 are electrically tunable lenses (Optotune)14, which provides a z-range of ~450 μm.

A scan lens and tube lens formed a 4× telescope (Supplementary Fig. 5) and relayed the expanded beams (24 mm) to the back aperture of the custom objective (effective focal length (EFL) = 27.5 mm; NA = 0.43; Supplementary Fig. 6). The entrance scan angles at the objective back aperture were ~± 3 degrees yielding our 3.5 mm FOV. Overall, the system transmitted 41% of the counter signals to a second Nuclear Instrumentation Module (NIM) discriminator (161L dual discriminator, LeCroy) (Supplementary Fig. 1b), which has a continuously potentiometer adjustment to adjust the output nuclear instrumentation module (NIM) pulse width from ~5 ns to >150 ns. This output pulse was delayed to the common veto input on the LeCroy 4608C discriminator where the PMT outputs were collected. The veto width was adjusted by the potentiometer on the LeCroy 161L discriminator and the relative phase of the veto window was adjusted by delaying the synchronization pulses from the laser module using small lengths of cables (~0.5 ns resolution). Two photon detection schemes were used. The first directly counts output pulses32 (digital counting) and the second is an analog counting scheme33. To implement digital counting, the demultiplexed output pulses from the LeCroy 4608C were sent to a NIM-to-TTL converter (PRL-350-TTL-NIM, Pulse Research Labs) before going to the counter inputs on a fast counter (PCI-6110, National Instruments) (Supplementary Fig. 1b). Counter measurements were organized into images in the custom LabView software. Pixel dwell time was determined by setting the counting window time in software. The maximum count rate for the PCI-6110 is 20 MHz and, therefore, it is possible some pulses are missed, lowering the overall signal amplitude. The analog counting mode output from the LeCroy 4608C is passed through a low-pass filter (EF508, EF516; Thorlabs) before being digitized at 5 MHz (PCI-6110, National Instruments) or 25 MHz (ATS860, AlazarTech). Analog measurements were arranged into images with the indicated pixel count in the custom LabView software. In this manner we could demultiplex the single PMT output into two channels corresponding to the two excitation pathways (Supplementary Fig. 1c).

Scanning hardware and strategy. Multiple scanning strategies were employed in this work: raster scanning using galvanometer and resonant scanners, and arbitrary line scanning.

Galvanometer scanning. The galvanometer-based scanning was performed using 6 mm galvanometers (6210H, Cambridge). Angular positions of the galvanometer were controlled by sending analog commands (PCI-6110, National Instruments) to the galvanometer driver board. The common reference clock on the PCI-6110 was used to synchronize the analog out commands and either the counters (digital counting) or the analog digitizer (analog counting) on the PCI-610.

Resonant scanning. Only the analog counting scheme was used with resonant scanning. Resonant scanning was accomplished using a galvanometer scanner on the slow X axis (6210H, Cambridge) and a 4 kHz resonant scanner (CRS04K-S4-5-045) on the fast Y axis. The resonant scanner was controlled using a CRS driver (Cambridge). This driver outputs a 50% duty cycle pulse with the rising edge occurring every period (~4 kHz) thus outputting a rising edge every other line and a falling edge on the alternate line. This synchronization signal from the CRS driver was input to an Arduino Uno board that output a 5 s pulse on every rising and falling edge thus converting the signal to an ~8 kHz signal. This signal was input to the PCI-6110 as the clock for the analog output which drove the slow X-axis galvanometer. In this way the X and Y axes were synchronized. Additionally, the CRS driver synchronization pulse was used as a trigger for analog acquisition (ATS860, AlazarTech). After a trigger event occurs, a number of digitization events occur. This forms the pixels along a bidirectional line and the number is found by dividing the digitization frequency by the resonant scan frequency and subtracting the re-arm time. For example, in this system the resonant scanner had a measured frequency of 3,941.8 Hz and for a digitization clock of 5 MHz and a re-arm time of 64 clock ticks = 5,000,000/3,941.8 = 64 – 1,204. This number plus 16 must be divisible by 32 as required by the digitizer and thus is rounded down to 1,200. Therefore 1,200 pixels make up every two lines. Interlacing is accomplished by finding the best line alignment offline and then subsequently using these settings online. Because of the small re-arm time of the digitizer, some pixels are lost at the edge of every even line (~70). These are given a value of 0 in the reconstructed image. As higher digitization frequencies are used, the
percentage of these pixels becomes less. For digitization at 25 MHz, every two lines contain ~6,256 pixels and the even lines have ~86 pixels given a value of 0. Additionally, for image synchronization, we use the counter on the PCI-6110 to output a pulse every # lines/2 pulses of the CRS synchronization signal. This is used as an arm-trigger input into the ATS860 board. These signals ensure synchronization between the galvanometer scanner, resonant scanner, and the digitization board. An additional analog output on the PCI-6110 is used to control the amplitude of the resonant scanner. Corrections for the sinusoidal scanning path were performed online in software 34.

Arbitrary line scanning. The line scan pathway was drawn in custom LabVIEW software and converted to voltage commands for the galvanometer scanners. The imaging rate (28.7 Hz; Supplementary Fig. 13b) was determined to yield approximately equivalent pixel dwell time as the slower raster scans.

Cross-talk. Many factors affect the cross-talk between the two pathways including GCaMP6 fluorescence lifetime, veto pulse width, veto pulse phase, PMT gain, and excitation power. The veto width was set to inhibit pulses arising from the off-target pathway while maximizing the signal from the desired pathway. The working value is ~6.25 ns though small deviations from this made little difference. The phase of the veto (inhibit counting) signal relative to the PMT signal played a more important role and was adjusted to give maximal signal in one channel, arising from the non-delayed excitation path (pathway 1), and maximal signal in the other channel, arising from the 6.25 ns delayed excitation path (pathway 2), while at the same time minimizing the signal from pathway 1 excitation when pathway 2 was blocked and similarly for pathway 2 into pathway 1 (Supplementary Fig. 2a). With increases in the laser power and decreases in gain, the effective cross-talk was greatly diminished (Supplementary Fig. 2a–c) owing to changes in the instrument response function (IRF). By adjusting the laser intensity and gain on the PMT, we can minimize the level of cross-talk, while still obtaining high signal-to-noise measurements of fluorescence transients. For the experiments shown in this study, the laser power was <200 mW out of the front of the objective (also see In vivo two photon imaging in Methods) yielding <5% cross-talk between pathways.

For cross-talk to influence the acquired Ca2+ imaging and measured correlations there must be an ROI identified in the off-target pathway that is in the same XY position as a true ROI (neuron) in the on-target pathway. False ROIs can occur, and these are ROIs that arise from the off-target pathway and are solely due to cross-talk. These can be identified by correlations in neural activity Ca2+ transients that satisfy two criteria: (1) have a high degree of correlation (>0.5), and (2) occur in the same XY locations in both images (pathway 1 and pathway 2). Thus, identification of the false ROIs is computationally simple, and they can be automatically removed from further analysis. Cross-talk was not detected in the data set displayed in Figure 4. A more subtle form of cross-talk can occur when two neurons, one in each pathway, are scanned simultaneously. To determine whether this form of cross-talk is affecting cross-correlation measurements, we searched the data set for pairs of neurons, one in each pathway, that overlap in XY position, and thus are simultaneously scanned and could contaminate each other’s signals. For these pairs, we computed the correlation between amount of overlap (the intersection of XY pixel locations between the two neurons’ ROIs) and activity cross-correlation. If activity signals from one neuron are contaminating the other, there should be a trend for more overlapped neurons to also exhibit higher cross-correlations. However, this relationship was not significant ($R^2 = 0.0094$, $P = 0.55$, $N = 40$ pairs), and thus even when cells are simultaneously scanned, cross-talk is low and high-fidelity measurements of neural activity are obtained.

Excitation PSF and individual neuron resolution. To compare the effect of different axial PSFex on acquired data, we simultaneously imaged GCaMP6s activity with two different excitation PSF (ref. 16: 14.0 ± 0.3 µm and 4.4 ± 0.2 µm (Supplementary Fig. 9a). To establish the two different PSFex, we used a Nikon 16x objective (NA = 0.8) and underfilled the back aperture of the objective using pathway 1 (14.0 ± 0.3 µm, which is slightly larger than that obtained in the Trepan2p), and overfilled the back aperture of the objective using pathway 2 (4.4 ± 0.2 µm). The PSFex measurements were made as described above (see Excitation point spread function measurements). The XY locations of the two pathways were slightly offset in order to ensure cross-talk due to multiplexing (though minimal) did not influence our measurements and to minimize the potential photo-damage. The two pathways scanned (~3 frames/s) the same neurons in V1 (Supplementary Fig. 9b) whereas the mouse was shown the naturalistic video. Calcium transients were extracted from the same cells in both pathways from three different fields of view (Supplementary Figs. 9c and 10). The correlation coefficients ($n = 456$ cells) were calculated between the two different excitation PSF pathways for the raw $\Delta F/F$ traces (Supplementary Fig. 9d).

These findings agree with a prior report16, which showed that in mammalian neocortex (imaging signals from pyramidal neurons expressing GCaMP6s), excitation PSF that are ~10 µm in the axial dimension can be sufficient to keep potential cross-talk between cells <1%. They found that although cross-talk can occur, in practice, the signals are rarely and minimally contaminated. Nearby cell bodies could conceivably contribute to cross-talk, but typically do not. This is because in mouse cortex, <10% of neurons are located axially <30 µm from each other16. Thus, the Nyquist criterion for keeping maximal potential cross-talk below 10% is an axial resolution that is <15 µm. In summary, as the vast majority of neurons are separated axially, cross-talk due to PSFex that are ~10–15 µm in the axial dimension do not preclude single neuron resolution.

In addition to the spatial distribution of neurons, these results can also be influenced by the staining pattern and the fluorescence of the indicator in the low calcium state. Thus other indicators, staining patterns, and imaged structures can impose different requirements on the imaging system.

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