Kilohertz two-photon brain imaging in awake mice

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Two-photon microscopy is a mainstay technique for imaging in scattering media and normally provides frame-acquisition rates of ~10–30 Hz. To track high-speed phenomena, we created a two-photon microscope with 400 illumination beams that collectively sample 95,000–211,000 µm² areas at rates up to 1 kHz. Using this microscope, we visualized microcirculatory flow, fast venous constrictions and neuronal Ca²⁺ spiking with millisecond-scale timing resolution in the brains of awake mice.

Two-photon fluorescence microscopy is widely used for imaging in turbid media, but its conventional laser-scanning form has limited speed ill-suited for tracking 1-ms scale events. Several past efforts explored fast laser-scanning mechanisms, but 1-kHz imaging via rapidly scanning one laser focus often yields poor fluorescence counts, as illumination intensity is limited by sample heating, photobleaching and phototoxicity. The resulting paucity of fluorescence photons can lead to a temporal resolution and event timing accuracy that are substantially poorer than the 1-ms resolution nominally implied by kilohertz image acquisition.

To boost fluorescence for fast two-photon imaging, one can reduce the number of pixels sampled, such as via sculpted illumination or random-access sampling. However, these approaches sacrifice micrometer-scale, full-frame imaging and impede imaging of particle trajectories or tissues requiring motion artifact corrections. Other approaches concurrently illuminate multiple pixels, such as with a line of illumination, which speeds up imaging but degrades optical sectioning, or with widefield two-photon excitation and temporal focusing, which maintains sectioning but degrades excitation efficiency. Another strategy involves multiple laser beams and multi-pixel detectors for concurrent sampling from multiple foci. This approach can greatly increase the emission of fluorescence photons, which are crucial for millisecond-scale temporal resolution. However, background fluorescence, from fluorescence scattering and interference between beams that excites fluorescence outside the focal plane, is a key design challenge. Overall, no previous study has demonstrated full-frame, in vivo two-photon imaging with sufficient fluorescent photons for millisecond-scale resolution and timing accuracy of biological events.

Here, we report fast two-photon imaging in awake mice up to 300 µm into tissue. Using a unique mechanism for scanning multiple beams, our microscope provided 1-kHz videos in live animals, for tracking microcirculation and ~10-ms resolution of neural Ca²⁺ transients. Key features are an ultrafast laser (pulses of 1,030 nm, ~10–30 µJ energy, ~280 fs duration, 0.2–2 MHz rate), an extra-cavity

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pulse picker, a microlens array creating a grid of laser beamlets, a scanning mirror and an sCMOS camera (100–1,000 frames per second) (Fig. 1a and Supplementary Figs. 1 and 2). To increase fluorescence, we used 50–200 kHz pulse picking and 10–19 J pulses, which enhanced excitation over conventional two-photon imaging while maintaining cell health (0.3–2.9 mW per focus).

We shaped the laser beam to a spatially uniform intensity at the microlens array, yielding an N × N array of beamlets of equal power (Supplementary Fig. 3). We generally used 20 × 20 arrays, tilted 2.9° = tan−1(1/N) relative to the scanning axis and camera image frame, with each beamlet sampling one image row (Fig. 1b). Adjacent laser foci were 15 μm apart in the specimen, yielding 0.75 μm between adjacent scanning lines. The point spread function at each focus had lateral and axial full width-half maximum (FWHM) values of 1.4 ± 0.1 (s.d.) μm and 9.6 ± 2.1 μm, respectively, suitable for the spacing of adjacent image lines. The camera acquired fluorescence images of 1,024 × 196 pixels (~95,000 μm² specimen area) at 1 kHz, or 1,024 × 512 pixels (~248,000 μm²) at ≤400 Hz, whereas the beamlets covered ~705 × 300 μm² for scanning rates ≤1 kHz. A fast camera with a larger sensor would allow 1-kHz imaging across the entire illuminated area.

To assess imaging in live mice, we studied cerebral arterioles with flow speeds up to ~12 mm s⁻¹. Past two-photon imaging studies of microcirculation used laser line scanning but have not tracked individual cells via high-speed full-frame imaging. We intravascularly injected fluorescently labeled human embryonic kidney (HEK-293T) cells, followed cells with millisecond-scale resolution (100–1,000 Hz imaging, eight mice, see Supplementary Videos 1 and 2), and mapped cells’ speeds (Fig. 2a–c). Different cells in the same vessel had distinct kinematics, especially in larger arterioles (Fig. 2d). Speeds rose quadratically with distance from vessel walls, as fluid mechanics predicts (Fig. 2e). With 1-kHz imaging, we observed the heartbeat’s influence (~150 ± 20 beats min⁻¹) on flow (Fig. 2f–h and Supplementary Video 2). In individual arterioles, flow speed declined in vessel portions with larger diameters (r = –0.61 ± 0.27 (s.d.); nine vessels in six mice), although larger vessels generally exhibited faster speeds than small ones (r = 0.60 ± 0.07).

Next, we studied bridging veins near the superior sagittal sinus (SSS). The cerebral venous system was thought to be primarily a passive drain, but smooth muscles at the SSS junction with bridging veins (Fig. 2i) suggest active mechanisms. To explore, we intravascularly injected a fluorescent dye and performed high-speed imaging in awake mice (Fig. 2i–k). At onsets of vessel constriction, bridging veins decreased in diameter (~6.5 ± 0.9% (s.e.m.) per 100 ms, 36 constriction events) faster than diameter increases at dilation onsets (~4.0 ± 0.7% per 100 ms, 36 dilations). Anesthetized mice lacked these high-speed vessel dynamics.

Next, we examined neural activity. We combined 1-kHz two-photon Ca²⁺ imaging in live cortical tissue slices with electrical measurements of neural membrane impedance and spike duration, and identified imaging conditions with minimal impact on membrane integrity, an indicator of neural health (Supplementary Fig. 4). During dual Ca²⁺ imaging and patch-clamp recordings, we electrically evoked single or trains of neural spikes to elicit Ca²⁺ transients. Spike trains of 10 Hz induced stepwise increments in fluorescence intensity (Fig. 3a and Supplementary Fig. 5). By comparing electrical and optical traces, we found that spike or spike burst initiation times can be estimated from the optical traces to ±11.6 ms (s.d.) (Fig. 3b). Computational studies verified 1-kHz imaging yields millisecond-scale accuracy (Supplementary Fig. 6).

We next studied awake mice expressing the Ca²⁺ indicator GCaMP6f in layer 2/3 cortical neurons. Fast two-photon imaging (100–1,000 Hz) yielded single cell Ca²⁺ activity traces up to ~300 μm into tissue (Fig. 3c–g and Supplementary Video 3). With 1-kHz
Two-photon imaging using the Ca\(^{2+}\) indicator Calbryte-590 (red traces, 100 Hz laser pulse rate, 0.7 mW per beamlet) of dendritic Ca\(^{2+}\) transients with asterisks in Fig. 3g. Transients from nine cells, aligned within 4.2 ± 2.4 ms (mean ± s.d., N = 4 transients) to the onset of excitation, are shown down-sampled to 500 Hz and median filtered (time constant, 16 ms). Asterisks mark individual Ca\(^{2+}\) transients shown in color-corresponding traces in f, g, h, i. Individual (colored traces) and mean (black trace) waveforms of four Ca\(^{2+}\) transients with asterisks in d, aligned within 4.2 ± 2.4 ms (mean ± s.d., N = 4 transients) to the onset of excitation. Magnified view of the gray-shaded portion of the marked Ca\(^{2+}\) transient in cell (l). Example 100-Hz Ca\(^{2+}\) imaging (200 kHz laser pulse rate, 2.9 mW per beamlet). Scale bar, 40 μm. d, h, i. 100-Hz Ca\(^{2+}\) imaging (200 kHz laser pulse rate, 2.9 mW per beamlet) of dendritic Ca\(^{2+}\) spiking activity of cerebellar Purkinje neurons expressing R-CaMP2 in awake mice. Example ΔF/F traces, h, of 25 neurons whose contours are shown in i superposed on a mean (0.5-min average) two-photon image. Gray shading in h marks four example events when ≥80% of the visible neurons spiked synchronously. Scale bar in i, 50 μm. Field of view, 450 × 300 μm.

J. Waveforms of 492 individual Ca\(^{2+}\) spikes, after alignment of baseline fluorescence levels and spike occurrence times. Red traces, 177 randomly chosen spikes from 23 neurons imaged as in h. Green traces, 315 randomly chosen spikes from 43 Purkinje neurons imaged using GCaMP6f and conventional two-photon microscopy (10-Hz imaging, 920 nm illumination, 30 mW). k, Scatter plot of the spike-timing estimation accuracy versus d′, the spike detection fidelity\(^{13}\), for each individual Purkinje neuron (red data points for cells studied by 100-Hz imaging, green points for cells studied by conventional two-photon imaging). Dashed curves represent theoretical limits on spike-timing accuracy for 100-Hz imaging (red curve) and 10-Hz imaging on the conventional two-photon microscope (green curve), computed using the Chapman–Robbins lower bound on the variance of an unbiased estimator\(^{13}\). Error bars, s.e.m. on all spikes recorded for each cell. l, Histogram of timing jitters for individual spikes in synchronous spiking events, where the jitter is the difference between each spike’s occurrence time and the mean time for all spikes in the synchronous event. Inset, expanded view of the histogram for synchronous spikes recorded by high-speed imaging. Error bars, s.d. estimated as counting errors. a, Shows traces representative of eight cells recorded in neocortical slices from three mice. c–g, Show data representative of that taken at six fields of view in each of three mice and h and i show representative results from 1-2 fields of view in each of four mice.

Imaging, rising phases of large-amplitude Ca\(^{2+}\) transients exhibited stepwise increments, consistent with fluorescence waveforms evoked in vitro by brief spike trains (Fig. 3g). By fitting the mean Ca\(^{2+}\) transient waveform to the activity traces, we estimated Ca\(^{2+}\) transient occurrence times to within ±12.8 ms (95% confidence interval (CI)).

To further study spike timing, we examined complex spiking by cerebellar Purkinje neurons in awake mice. To visualize the spike’s dendritic component, we used R-CaMP2, a red Ca\(^{2+}\) indicator suited to 1,030-nm illumination. Using 100-Hz imaging, we tracked tens of cells concurrently and focused on dendrites ~100–200 μm below the brain surface (227 cells, four mice, see Fig. 3h,i). We acquired 30-s Ca\(^{2+}\) videos spaced ~2 min apart over ~25–30 min, computationally extracted individual cells and their Ca\(^{2+}\) spiking activity, and evaluated the spike-timing precision against that of conventional two-photon microscopy.

For stringent comparisons, our conventional two-photon imaging (10 Hz) used the Ca\(^{2+}\) indicator GCaMP6f, which has comparable kinetics and greater dynamic range (ΔF/F) than R-CaMP2. To optimize GCaMP6f emission we used 920-nm illumination (20–35 mW). Ca\(^{2+}\) spiking rates under conventional (0.5 ± 0.1 (s.d) s⁻¹) and high-speed (0.6 ± 0.1 s⁻¹) microscopy were indistinguishable (227 cells). But, even with the superiority of GCaMP6f, high-speed imaging with R-CaMP2 better revealed the fluorescence waveforms (Fig. 3i). For instance, the mean rise time to half-maximum amplitude was 13 ± 5 (s.d.) ms for 177 R-CaMP2 spikes imaged at 100 Hz and 50 ± 15 ms for 315 GCaMP6f spikes imaged at 10 Hz.

For each cell, we computed the spike-timing accuracy and detection fidelity using the metric d′ from signal detection theory\(^{13}\) (Fig. 3k). Even with the ~40% smaller amplitude of R-CaMP2 spike waveforms versus those of GCaMP6f, the greater fluorescence flux...
of the high-speed microscope yielded ~50% greater \( d' \) values. After fitting a parameterized waveform to the fluorescence trace of each Ca\(^{2+} \) spike, we determined the spike-timing estimation accuracy as the 95% CI for the spike occurrence time. This yielded accuracies of 6.8 ± 3.4 ms (mean ± s.d.) and 48 ± 30 ms, respectively, for high-speed (100-Hz imaging, 177 R-CaMP2 spikes) and conventional (10-Hz imaging, 315 GCaMP6f spikes) two-photon microscopy. We compared these values to the theoretical limits set by the Chapman–Robbins lower bound on the variance of an unbiased estimator\(^{13} \) and found that high-speed Ca\(^{2+} \) imaging allows timing accuracies within several milliseconds of the physical limitations (Fig. 3k). As nearby Purkinje cells often fire synchronized complex spikes\(^{14} \), we also analyzed the timing jitter between synchronized spikes. The jitter observed by 100 Hz imaging was 7.8 ± 5.5 ms (s.d.), versus 61 ± 53 ms by conventional imaging, which thus inflated the jitter by ~800% (Fig. 3i).

Overall, our microscope allows ~30–100× faster imaging than conventional two-photon microscopy. By capturing sufficient numbers of fluorescence photons, our system provides millisecond-scale temporal resolution and timing accuracy, which had not been demonstrated in live animals for previous two-photon microscopes with kilohertz, full-frame image-acquisition rates. We thereby revealed microcirculation, rapid vessel constrictions and the fine timing of neural activity. Photodamage was reduced by the use of long-wavelength illumination (1,030 nm), the low power (≤3 mW) and 15-μm-spacing of adjacent foci, and pulse picking to minimize tissue exposure. Imaging bouts of around 30 s were spaced ~2 min apart, allowing time for cells to recover and new fluorophores to reach the focal plane. In neuroscience research, animals often perform ~100 or more behavioral trials across a 1-h session, with each trial lasting seconds\(^{15} \). With this design, ~3 s of high-speed imaging per trial would keep brain temperatures within physiological ranges, even at the maximum powers used here (Supplementary Fig. 7).

Faster scientific cameras, larger illumination arrays and faster scanners could further improve imaging speeds and fields of view. We imaged ≤300 μm into the brain, beyond the ~75–100-μm attained with planar illumination microscopy\(^{2,15} \). Camera frame-rates >1 kHz, combined with image subsampling and computational un-mixing of fluorescence scattering, could potentially improve imaging depths. To illustrate, we studied scattering patterns in fixed tissue and used this information to visualize cells up to ~500 μm deep (Supplementary Figs. 8 and 9). Further reduction of background fluorescence, excited outside the focal plane due to interference between beamlets, is also feasible (Supplementary Fig. 10). Thus, we expect multi-focus, high-speed two-photon imaging will continue to improve for visualizing millisecond-scale biological events.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at [https://doi.org/10.1038/s41592-019-0597-2](https://doi.org/10.1038/s41592-019-0597-2).

Received: 4 September 2016; Accepted: 11 September 2019; Published online: 28 October 2019

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**Acknowledgements**

We thank Y.-S. Chen, E. Cocker, A. Fritz, K. Ghosh, B. Grewe, E.T. Ho, J.C. Jung, H. Kim, J. Lecoq, J. Li, J. Lu, J. Marshall, O. Rumyantsev, G. Sanchez, J. Savalli, S. Sinha, T. Tasci, D. Vacirce, B. Wilt, G. Yin and B. Zhang for conversations and assistance, and K. Merkle for machining. M.J.S. received funds from the FG. Allen Family Foundation, NSF Neuronex grant no. 1707261, and the HHMI. H.B. was funded by JST-CREST, AMED-Brain/MINDS and KAKENHI research grant nos. 16K13105, 17K19442 and 17H06312.

**Author contributions**

T.Z. designed the microscope. T.Z., O.H. and R.C. built the microscope. T.Z., O.H., R.C., Y.Z., M.J.W. and I.Z.L. did imaging studies. O.H. and Y.Z. studied brain temperature. A.S. and O.H. performed patch-clamp studies. R.C. studied fluorescence scattering. T.Z., O.H., R.C., A.S. and B.A. analyzed data. M.I. and H.B. provided R-CaMP2. H.Z. provided transgenic mice. T.Z., O.H., A.S., R.C. and M.J.S. wrote the paper. M.J.S. supervised the project.

**Competing interests**

T.Z. and M.J.S. patented the microscope. M.I. and H.B. filed a patent on R-CaMP2.

**Additional information**

Supplementary information is available for this paper at [https://doi.org/10.1038/s41592-019-0597-2](https://doi.org/10.1038/s41592-019-0597-2).

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**Peer review information**

Nina Vogt was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

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Methods

Mice. The Stanford University Administrative Panel on Laboratory Animal Care approved all procedures using animals. For studies of neuronal hemodynamics, we used male mice (10–12 weeks old) that were either of the C57BL/6J (Jackson Labs) or PV-Cre/Ai14 mice, which express the red fluorescent protein tdTomato in parvalbumin interneurons. For imaging studies of layer 2/3 cortical pyramidal neurons in live mice, we used triple transgenic GCAMp6f-ATA-dCre (Ragf2-2A-dCre, Camk2a-ATA, Ai93) mice from the Allen Institute (10–16 weeks old at the time of surgery). For studies of cerebellar Purkinje neurons, we used male and female PCP2-Cre driver mice (8–16 weeks old), which express Cre recombinase in Purkinje cells. Studies in fixed brain slices of neurons expressing Camk2a-tTA × Rasgrf2-2A-dCre (Jackson Laboratory) or PV-Cre/Ai14 mice, which express the red fluorophore tdTomato, were performed using male mice (10–12 weeks old) that were either of the C57BL/6J strain or male and female PCP2-Cre driver mice (8–16 weeks old), which express Cre recombinase in Purkinje cells. Studies in fixed brain slices of neurons expressing yellow fluorescent protein (YFP) or tdTomato were performed using C57BL/6J male mice from Jackson Laboratories (2–6 weeks old).

Microscope design. We performed optical design studies using Zemax software (Zemax Development Corporation) and mechanical design studies using Solidworks 2012 (Dassault Systèmes). A diode-pumped Yb+ ion ultrashort-pulsed laser amplifier (Tesseract, AdOptica) was combined with a prohibit beam splitter cube (PBS103, Thorlabs). We remotely controlled the translation of the half-wave plate using a motorized precision rotation stage (PRM1Z8E, Thorlabs). We remotely controlled the rotation of the half-wave plate using a motorized precision rotation stage (PRM1Z8E, Thorlabs). We remotely controlled the rotation of the half-wave plate using a motorized precision rotation stage (PRM1Z8E, Thorlabs). We remotely controlled the rotation of the half-wave plate using a motorized precision rotation stage (PRM1Z8E, Thorlabs).

We made fine adjustments in laser illumination power by changing the modulation efficiency of the pulse picker within the laser.

We used a custom beam-shaping subsystem (Fig. 1a, inset) that consisted of a variable beam expander (68–481, Edmund Optics), a flattop beam shaper (pShaper 6_6_xTIS, AdlOptica) and a custom Keplerian beam reducer made of a 1-mm thick steel chopper wheel with ten uniform blades that blocked the laser beam with a 20% duty cycle. The chopper resided upstream of the laser beam with a 20% duty cycle. The chopper resided upstream of the laser beam with a 20% duty cycle. The chopper resided upstream of the laser beam with a 20% duty cycle. The chopper resided upstream of the laser beam with a 20% duty cycle. The chopper resided upstream of the laser beam with a 20% duty cycle. The chopper resided upstream of the laser beam with a 20% duty cycle. The chopper resided upstream of the laser beam with a 20% duty cycle.

We generally configured the optical pathway in one of two different ways, termed Configurations 1 and 2. Both configurations yielded a 15-µm separation between the laser microbeams in the specimen plane. We performed nearly all imaging with Configuration 1 (Figs. 1c–1e and Supplementary Figs. 3 and 4) and later switched to Configuration 2, which exhibited greater stability (Fig. 3a,b and Supplementary Fig. 5). In Configuration 1, the scanning mirror and a set of aspheric, polymer-coated achromatic lenses (49–665, 46–665 and 49–664, all Edmund Optics) for lenses L2, L3 and L5, respectively, in Fig. 1a) were positioned such that the scanning mirror and the back aperture of a water-immersion microscope objective lens (a Olympus XUMPLFNW-0.95 NA x20 lens, a Olympus XUMPLFNW-1.0 NA x20 lens or a Nikon NIR APO x40 0.8 NA, 3.5-mm working distance, used for all in vitro patch-clamp experiments) were located in optical planes that were Fourier-conjugate to the specimen plane. A pair of custom, compound meniscus lenses (1.4 and 1.4 in Fig. 1a, each composed of two singlet lenses (48–710 and 49–516, Edmund Optics)) were held with opposing orientations in the optical pathway and thereby alleviated the field curvature of the laser focal plane in the specimen.

In Configuration 2, we placed the following achromatic lenses between the microscope objective lens and the square grid of beamlets: a lens with a 3-cm focal length (AC254-030-B-ML, Thorlabs) and a lens with a 5-cm focal length (AC254-050-B-ML, Thorlabs) resided between the scanning mirror and the objective lens.

We often used a slit in the intermediate image plane lying after the microlens array, as well as an iris in an intermediate plane after the scanning mirror, to restrict the illumination beam pattern to the region imaged by the sCMOS camera such that the image line sampled across the camera region by less than 5% of its full horizontal extent. Thus (Fig. 1b), the fraction of the scanning cycle during which each image line was illuminated was less than the 80% of the scanning cycle during which laser illumination passed through the chopper wheel or pulse picker.

A dichroic mirror (FF705-D01-25x56 in Configuration 1 or DD03-R755-13-25x56 in Configuration 2, both from Semrock) reflected fluorescence emissions from the specimen to the microscope’s collection pathway. A fluorescence emission filter (FF61-505/40-25 or FF550-D05-20x5-25x6, Semrock) blocked light of wavelengths outside the emission pass band. After passing through the emission filter, in Configuration 1 the fluorescence photons passed through a tube lens (1.6 in Fig. 1a; 32–984, Edmund Optics) and an achromatic relay lens pair (Maxiphase 1000–A, Thorlabs). In Configuration 2, we used a variable focal length (55–200 mm) photography lens (Nikon, f/4–f/5.6) in the fluorescence collection pathway that allowed us to vary the magnification of the fluorescence image on the sCMOS camera (Fig. 1). A high-speed sCMOS camera (ORCA Flash4.0, Hamamatsu or Zyla 4.2 PLUS, Andor; 1–2 electrons r.m.s. read noise per pixel) captured the fluorescence images. The camera’s maximum data throughput was a bottleneck limiting the sizes of images that could be acquired at high speeds.

We used a digital signal output from the laser that was synchronized with pulse emission as an external clock for a multifunction data acquisition card (PCI-6110, National Instruments). This card sent synchronized timing signals to the laser-source in mirror, sCMOS camera and optical chopper system (Supplementary Fig. 2). To control the microscope, we created a custom software interface using the LabVIEW programming environment (National Instruments). During data acquisition, the data streamed right to disk. Beforehand, the user could preview the high-speed videos, but these preview data were not saved.

When imaging at frame-acquisition rates of 100–400 Hz (Fig. 2a,b, and Figs. 3a–d), we set the frequency of the scanning mirror to equal the frame-acquisition rate of the sCMOS camera, enabling uniform illumination across successive image frames. The field of view for images acquired at ≤400 Hz frame rate was ≤705 × 300 µm², corresponding to 1,024 × 436 camera pixels. When the imaging frame rate was 1 kHz, we set the frequency of the scanning mirror to 500 Hz and acquired separated frames on the forward and reverse portions of the scanning motion. Due to the limitations on data throughput set by the camera, the field of view when imaging at a 1 kHz frame rate was ≤705 × 135 µm², corresponding to 1,024 × 196 camera pixels. In all cases, the laser pulse repetition rate was 200 kHz, with a pulse-picking frequency between 50 and 200 kHz.

The above frequencies of laser pulse emission and image frame acquisition were chosen such that every pixel in the acquired image was illuminated by at least one laser pulse per frame. However, pulse emission was not synchronized to the passage of the laser beamlets across the specimen sampled by individual pixels on the camera. Thus, to account for the residual spatial nonuniformities of illumination, for each imaging configuration we performed a calibration in which we imaged a uniformly illuminated fluorescent reference slide. The image of this slide provided a map of illumination intensity, which we used to correct all subsequent images by normalizing each pixel’s emission intensity value by its level of illumination.

In vitro whole-cell patch-clamp electrophysiological recordings. To verify that the illumination protocols used for high-speed two-photon imaging did not impair cell health, we performed whole-cell patch-clamp recordings in live neocortical tissue slices from PV-Cre/Td-Tomato mice (4–8 weeks old) simultaneous with high-speed two-photon imaging.

We performed the mice with artificial cerebrospinal fluid (ACSF: 92 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 25 mM glucose, 5 mM sodium ascorbate, 2 mM threonine, 3 mM sodium pyruvate, 10 mM MgSO4, 0.5 mM CaCl2). After perfusion, we excised the brain and made tissue slices (300 µm thick) using a tissue slicer (Leica VT-1200S) while the brain was immersed in ACSF. After making six coronal slices, we incubated the slices at 34 °C for 10 min and then transferred them to a room temperature ACSF of identical composition to above except with 2 mM MgSO4 and 2 mM CaCl2.

We performed whole-cell patch recordings of tdTomato-positive neurons while the tissue slices were immersed in recording solution (124 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 24 mM NaHCO3, 20 mM HEPES, 25 mM glucose, 5 mM sodium ascorbate, 2 mM threonine, 3 mM sodium pyruvate, 10 mM MgSO4, 0.5 mM CaCl2). We used glass recording pipettes (pulled to 4–7 MΩ impedance with a laser puller (Sutter P-2000)), filled with intracellular solution (125 mM potassium chloride, 10 mM KCl, 10 mM HEPES, 40 mM MgATP, 0.3 mM NaGTP, 10 mM Na2phosphocreatine, 1 mM EGTA).

We tested the health of the patched pyramidal neurons by measuring their membrane impedance values and action potential durations across imaging sessions of 30-min duration that alternated between 30-s periods of two-photon imaging and 2 min of recording (Supplementary Fig. 1). We examined two different regimes, which typified the conditions we generally used for high-speed brain imaging in live mice. In the first regime, we used illumination pulses of high peak intensity (100 kHz pulse repetition rate, 0.53 µm² mean beamlet power, 400 Hz imaging frame rate). In the second regime, we studied a higher mean...
illumination power but with individual pulses of lower peak intensity (1 MHz pulse repetition rate, 2.3 mW average power, 100 Hz frame rate). To assess the accuracy of spike-timing estimation based on neuronal Ca\(^{2+}\) activity, we chose to obtain high-speed two-photon imaging since we performed in vitro whole-cell patch-clamp recordings in live cortical slices with concurrent high-speed two-photon microscopy. Following the procedures described above, we made cortical slices from male C57BL/6J wild type mice (2–6 weeks of age). In these experiments, we filled neurons with an intracellular solution containing 50 mM of the Ca\(^{2+}\) indicator Calbryte-596 (AAT Bioquest). We patched single neurons and evoked either single spikes or bursts of action potentials using one or a train of 2-ms current pulses delivered at either 150 or 10 Hz (Supplementary Fig. 5a).

**Surgical procedures.** To prepare mice for in vivo imaging sessions, we performed surgeries using isoflurane anesthesia (1.5–2% isoflurane in O\(_2\)) while mice were mounted in a stereotactic frame. To reduce post-operative inflammation and pain, we administered a pre-operative dose of carprofen (5 mg kg\(^{-1}\), subcutaneous injection into the mouse’s lower back), which we repeated once a day for 3 d following the surgery.

For studies of neocortical microcirculation (Fig. 2a–h), we created a cranial window by removing a 5-mm diameter skull flake (centered at AP (anterior–posterior) −2.5, ML (medio–lateral) 3.0) or a 7-mm diameter skull flake (centered at AP −1.0, ML 0.0). We covered the exposed cortical surface with a glass cover slip (thickness no. 1), which we secured to a thin plate using an ultraviolet-light curable cyanoacrylate glue (Loctite 4305). We cemented a metal head plate, and placed the mouse on a running wheel under the objective lens of the two-photon microscope. Two-photon brain imaging sessions lasted 10–30 min. The mouse was free to walk or run in place during brain imaging.

To image neocortical pyramidal cells expressing GCaMP6f, we head-restrained mice with exposed glass cranial windows using two stainless steel bars that were affixed to the left and right sides of the head plate. The mice were placed on a running wheel under the microscope objective lens and allowed to run or walk in place. Imaging sessions alternated between imaging intervals (<30 s duration) and recovery intervals (2 min duration).

To image cerebellar Purkinje neurons expressing GCaMP6f, we used a custom-built two-photon microscope of a conventional design. A Ti:sapphire laser (SpectraPhysics, MA, USA) provided ultrashort-pulsed illumination (920 nm wavelength). Power at the sample was 20–35 mW. We used a 0.95 NA ×20 microscope objective lens (Olympus XLUMPlanFI). We acquired raw images across a field of view of 110 × 270 μm\(^2\) (130 × 280 pixels) at 20 Hz and downsampled them to 10 Hz.

**Measurements of brain temperature during high-speed two-photon imaging.** To perform temperature measurements in the brains of awake mice (GCaMP6f/i2TA-dCre) during high-speed two-photon imaging, we surgically prepared mice by performing a 5-mm diameter craniotomy following the same surgical procedures as for imaging studies of microcirculation (see above). However, before placement of the cranial window, we inserted a flexible 200-μm diameter thermocouple probe\(^{22}\) (TT24P, Physitemp) into the brain, 100–200 μm beneath the dura, near the edge of the craniotomy (Supplementary Fig. 7a). The thermocouple resin was cured over a 5-mm long plastic micropipette and extended −2.5 mm beyond the tip of the microcatheter.

Using ultraviolet-light curable glue (Loctite, 4305) and dental cement, we affixed the microcatheter to the cranium at a shallow angle of 5° relative to the surface of the cranium. We then placed the glass cranial window onto the craniotomy and fixed the window in place with dental cement. The thermocouple probe was connected to a temperature data logger (CTLS15R, Omega) that was exposed to the normal body temperature in mice, as previously reported\(^{18}\). After the onset of imaging, the time-courses of the temperature measurements, normalized by the illumination power, all had similar time-dependencies\(^{22}\) and were predictable in advance (Supplementary Fig. 7c,d).

**Blood flow speeds.** To determine the average speed of fluorescent HEK-293T cells through a chosen reference pixel in a fluorescence video, we computed the temporal cross-correlation between the fluorescence intensities at that pixel and at neighboring pixels, for relative temporal delays, τ, of −1, 0, and +1 image frames\(^{23}\). After setting to zero the values of all pixels lying outside blood vessels or exhibiting cross-correlations with the reference pixel below a minimum threshold value, we computed the centroid of each of the three cross-correlograms. We determined the mean speed of the cells passing through the reference pixel as the average of the two distances between the cross-correlation centroids from successive image frames, divided by the time between successive image frames. To determine the mean flow speed, we computed the extent to which each pixel was traversed by all pixels in a vessel. To create a time series of mean flow speed, we repeated the procedures above for a given region of interest in the video across time windows of a uniform duration.

To calculate the streamlines of individual cells’ trajectories, we computed a movie that equaled the difference of successive image frames in the raw movie data. To highlight the motion of individual cells, we applied both positive and negative binary thresholds to the difference movie. To remove noise from the resultant, we applied the ‘open’ then ‘close’ morphological steps in image processing\(^{24}\). We used the spatial average of the centroids of the positive and negative blobs as an interpolation of the cell’s position between successive image frames. After thresholding, we computed the streamlines by following the path of all pixels in a vessel. To create a time series of mean flow speed, we repeated the procedures above for a given region of interest in the video across time windows of a uniform duration.

**Analysis of blood vessel diameter.** To determine the width of a blood vessel in an individual image frame, we visually identified the region of interest and fit a Gaussian function to the fluorescence intensity values along a cross-section of the vessel’s centerline. To calculate the vessel diameter, we plotted the vessel diameter values as a function of time (Fig. 2b). We visually scored the onset times of constrictions and dilations by identifying local maxima and minima in the time traces of vessel diameter.

**Extraction of Ca\(^ {2+}\) activity traces.** To extract the somatic Ca\(^ {2+}\) activity of neocortical layer 2/3 pyramidal cells expressing GCaMP6f (Fig. 3d–g and Supplementary Video 3), we first filtered each movie frame using a band-pass Gaussian spatial filter (typical spatial cutoffs: 0.42–1.2 and 12–24 μm). To
extract Ca\(^{2+}\) activity traces, we manually encircled regions of interest that were \(\sim 12\mu m\) in diameter and plotted the mean fluorescence within these regions as a function of time.

To extract dendritic trees of individual Purkinje cells from in vivo two-photon Ca\(^{2+}\) imaging videos, we used an established cell-sorting algorithm based on the successive application of principal component analysis and independent component analysis\(^3\). We selected spatial filters provided by independent component analysis that corresponded to individual Purkinje cells, smoothed the filters by applying a one-round of spatial erosion and dilation\(^2\), and applied a threshold to obtain a final set of spatial masks that we applied to the fluorescence videos to obtain the Ca\(^{2+}\) activity-related traces from individual cells, \(\Delta F(t)/F_0 = (F(t) - F_0)/F_0\), where \(F_0\) is the mean value of the time series.

Identification of Ca\(^{2+}\) spikes. To identify Ca\(^{2+}\) spikes in the Purkinje cells’ activity traces, we first temporally deconvolved each cell’s \(\Delta F(t)/F_0\) trace with a decaying exponential function (150 ms time constant) to account for the signal decay kinetics of the Ca\(^{2+}\) indicator\(^-\).\(^-\). We then applied a threshold function to identify all positive-going signal deflections. We retained as spikes all such deflections that reached a peak value at least twice the standard deviation of the cell’s baseline fluorescence fluctuations.

Fidelity of spike detection. To estimate the fidelity of spike detection\(^1\), we calculated \(d'\) for each Purkinje neuron as \(d' = \frac{\langle \Delta F(t)/F_0 \rangle - \langle \Delta F(t)/F_0 \rangle_{\text{baseline}}}{\text{standard deviation of } \langle \Delta F(t)/F_0 \rangle_{\text{baseline}}}\), where \(\langle \Delta F(t)/F_0 \rangle\) and \(\langle \Delta F(t)/F_0 \rangle_{\text{baseline}}\) denote the fluorescence amplitudes of the Ca\(^{2+}\) spike and baseline fluorescence, respectively, and \(\text{standard deviation of } \langle \Delta F(t)/F_0 \rangle_{\text{baseline}}\) is the uncertainty in the occurrence time.

We then used the waveform of this parametric fit as a matched filter for estimating the spikes or spike burst occurrence time as the time bin at which the output of the matched filter attained a local maximum. We averaged these time points across all the neurons to obtain the mean time for all spikes in the synchronous event.

Collision of synchronous Purkinje cell Ca\(^{2+}\) spikes and computation of spiking jitter. We visually identified candidate occurrences of synchronous spiking, examined the occurrence times for all the constituent spikes and required that \(\geq 80\%\) of the cells must have spiked within \(\pm 1\) time bin from the candidate event’s mean spike time. For each event and its constituent spikes that passed this criterion, we determined the timing jitter of each spike as the difference between its occurrence time and the mean time for all spikes in the synchronous event.

Studies of fluorescence scattering. We used fixed coronal brain slices of the GCaMP6f-eta-dCre mice to assess the extent of fluorescence scattering for different imaging depths in tissue. Using a stationary laser beam to induce localized two-photon excited fluorescence at different depths in tissue, and the resulting fluorescence images captured by the sCMOS camera, we measured the lateral spatial distributions of fluorescence, \(P(x,y)\), relative to the position of the laser focus at \(x = y = 0\). We averaged these spatial distributions over all polar angles \(\theta\) and 50 different individual locations of the laser beam for each of 51 different depths in tissue and seven different cortical regions. We normalized \(P(x,y)\) so that its integral over all space was unity, allowing one to interpret \(P(x,y)\) as a probability density (Supplementary Fig. 8a–c).

To compute the percentage of fluorescence photons emitted from the focus of one laser beamlet that would scatter in tissue into a nearby image tile (Supplementary Fig. 8d), we used the xy coordinate system aligned with the grid of laser beamlets and applied the formula

\[
p_I = \int P_s(x,y) \, dx \, dy
\]

where \(P_s\) is the probability that a photon excited by the \((m,n)\)th beamlet will be detected in the \((n+1, m+1)\)th tile, and the domain of integration is given by \(\{x \in [n \pm 1, d/2, (n+1) \pm d/2], y \in [m \pm 1, d/2, (m+1) \pm d/2]\}\). Using this approach, we empirically determined \(p_s(x,y)\) and \(P_s\) for the protein fluorophore GCaMP6f (Supplementary Fig. 8a–d).

We also estimated the depth-dependent decline in fluorescence Ca\(^{2+}\) signals that would occur in a neural Ca\(^{2+}\) imaging study using GCaMP6f (Supplementary Fig. 8e). For each depth in tissue, we convolved the corresponding \(P_s(x,y)\) distribution with a uniform, circularly symmetric source of fluorescence that was \(12\mu m\) in diameter, about the size of a neocortical neural cell body. From the results of this convolution, we computed the proportion of fluorescence signals that remained inside the cell body perimeter. We fit these determinations of signal decline with imaging depth to an exponentially decaying function, \(e^{-d/\lambda}\), with a characteristic decay length of \(\lambda = 142 \pm 30\) μm (s.d., \(N=7\) different locations in tissue).

Image reconstructions using subsampling. For these proof-of-concept studies (Supplementary Fig. 9), we used an optical layout that was a re-arrangement of Configuration 2 (see above) with a \(24\mu m\) separation between the foci of adjacent laser beamlets in the specimen plane. Specifically, the 3-cm focal length (AC254-030-B-M, Thorlabs) lens resided between the micro lens array and the scanning mirror. The 0.3-cm focal length (AC254-040-B-M, Thorlabs) lenses resided between the scanning mirror and the objective lens. In the fluorescence collection pathway, we used a 8.5-cm focal length photography lens (Canon, f/1.8) as the tube lens. We explored two alternative image processing approaches based on image subsampling, as a means of counteracting the effects of fluorescence scattering in tissue.

In both cases, the image subsampling involves acquiring multiple camera frames at regular time intervals in a single scanning period of the galvanometer mirror. One can then computationally correct each image subframe for the effects of fluorescence scattering before reconstructing the final image. The first approach, termed ‘Reconstruction Method 1’ involves a spatial deconvolution of the image subsamples before assembling the final reconstructed image. The second approach, termed ‘Reconstruction Method 2’, involves a re-assignment of fluorescence signals to the nearest laser beamlet focus, followed by a spatial un-mixing of these signals to account for fluorescence scattering and then reconstruction of the final image.

For our studies of both reconstruction methods, we acquired 2,048 image subsamples per scanning cycle (800 × 1,024 pixels, 256 Hz camera-frame acquisition rate). We denote \(I(x,y)\) as the fluorescence image in the absence of any fluorescence scattering. We then approximate the image in the presence of fluorescence scattering, \(I_s(x,y)\), as a convolution (denoted by the operator, \(G\)):

\[
I_s(x,y) = G(I(x,y)) = \int I(x,y) \, dx \, dy
\]

The set of points at which the laser beamlets are focused is described by the function

\[
B(x,y) = \frac{1}{T_s} \sum_{\ell}[\delta(x - \ell d - x_\ell, y - \ell d - y_\ell)]
\]

where \(d\) is the spatial separation between adjacent beamlets, \(x\) and \(y\) are horizontal and vertical (integer) indices, respectively, \(x_\ell\) and \(y_\ell\) are spatial offsets, \(T_s\) is the time elapsed since the start of the scanning cycle, \(v_s\) is the speed at which the beamlets are swept across the specimen, and \(Q_s\) denotes the operator that rotates the image frame by...
it is well known that in practice this operation yields unsatisfactory results due to the severity and nonuniformity of fluorescence scattering deep in tissue. However, as shown in Supplementary Fig. 9, fluorescence scattering can be effectively counteracted if a deconvolution or un-mixing operation is applied to the individual subframes, \( I_n \), before image reconstruction.

**Reconstruction Method 1.** We reconstructed the final image by deconvolving the effects of scattering from every subframe, and then forming the maximum projection image across all the deconvolved subframes:

\[
I(x, y) \approx \max_n G^{-1}(I_n, P_n)(x, y)
\]

The maximum projection operation reduced the impact of background fluorescence, which arises from both fluorescence scattering and fluorescence excitation outside the focal plane (Supplementary Fig. 10). To perform the deconvolution, we used the MATLAB function deconvblind, with the kernel initialized as the function \( \exp(-\sigma^2 \cdot (x^2 + y^2)) \) with \( \sigma \) determined values for \( \gamma \), which accounted for the probability that a fluorescence photon had scattered from a plane beneath the tissue surface.

Suppressing optical interference between laser beamlets. The studies of Supplementary Fig. 10 demonstrate that the image contrast in high-speed two-photon microscopy can be enhanced by suppressing the homogeneous background fluorescence that arises from optical interference between the different beamlets and is consequently excited at out-of-focus Talbot planes and fractional Talbot planes\(^1\). As in previous work\(^2\), we sought to minimize such interference by desynchronizing the optical pulses within the different beamlets. However, we used a distinct strategy for achieving this desynchronization, in which we imposed a spatially pseudo-randomized set of temporal delays on the different beamlets (Supplementary Fig. 10a), rather than a spatially periodic set of delays as used in past work\(^3\).

We designed custom delay masks and had them commercially fabricated (Fluence Sp. z o.o.) by laser micromachining of glass plates (Supplementary Fig. 10c). Each mask comprised a stack of multiple glass plates, each of which was a 170-μm thick coverslip. Each plate contained a set of square holes, pseudo-randomly arranged in space. The sides of adjacent holes were separated by no less than one row or column of beamlets. The relative temporal delay between a beamlet traversing 170μm of glass and a beamlet traversing an air hole was 280fs. Thus, by stacking six glass plates in series, we achieved an assortment of different temporal delays ranging from 0–1,680fs in seven equal increments. Notably, our approach to desynchronization is scalable to larger arrays of beams and delay masks conferring a wide range of different delays.

To assess the benefits of this form of desynchronization, we imaged fixed neocortical tissue from GCaMP6f-TTA-dCre mice with and without insertion of the delay mask in front of the microlens array. The delay mask indeed reduced background fluorescence to an extent comparable to that achievable by low-pass spatial filtering of the images (Supplementary Fig. 10e).

We quantified the reduction in background fluorescence excitation that was attainable via laser pulse desynchronization. To do this, we placed a slide with a 1–2μm thick, uniform fluorescent layer (FluorCal FC-OCS-EC, Valley Scientific) into the path of laser beamlets, and we characterized the resulting patterns of fluorescence as a function of the axial displacement between the slide and the focal plane of the beamlet array, with and without insertion of the delay mask in front of the microlens array (Supplementary Fig. 10f). We also assessed how these measurements were affected by unlabeled brain tissue slices lying atop the fluorescent slide (Supplementary Fig. 10g). With brain slices of increasing thickness, the ability of the temporal delay mask to reduce background fluorescence declined in efficacy, suggesting that forward scattering in tissue of the excitation beams increases the extent of optical interference outside the focal plane.

**Statistical and image analyses.** We performed all analyses using custom software written in MATLAB (Mathworks). The Reporting Summary provides additional details.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The experimental data used for the analyses is available from the corresponding author upon reasonable request.

**Code availability**

The software code used for the analyses is available from the corresponding author upon reasonable request.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

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Data collection
- We acquired data using Labview (National Instruments; version 2016)

Data analysis
- We performed analyses using MATLAB (Mathworks; versions R2105b and 2017a).

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| Sample size | We used past work with similar specimens as a guide to determination of useful sample sizes. |
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| Data exclusions | There were no data exclusions. |
| Replication | The main findings of the paper all concern the imaging capabilities of the microscope, all of which were reproducible across multiple specimens. |
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| Blinding | N/A. There were no groups used in the study. |

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Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
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Eukaryotic cell lines

Policy information about cell lines

- Cell line source(s): TriBioScience Inc.
- Authentication: We labeled HEK-293T cells with fluorescent markers for our acute imaging studies of blood flow. We neither required nor performed an independent authentication of the cell line.
- Mycoplasma contamination: We labeled HEK-293T cells with fluorescent markers for our acute imaging studies of blood flow. We neither required nor performed testing for mycoplasma contamination.
- Commonly misidentified lines: We did not use any cell lines on the registry of commonly misidentified cell lines.

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Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

- Laboratory animals: For studies of cerebellar Purkinje neurons we used male and female PCP2-Cre driver mice (8–16 weeks old), which express Cre recombinase in Purkinje cells.

  For imaging studies of layer 2/3 cortical pyramidal neurons in live mice, we used male and female triple transgenic GCaMP6f-tTA-dCre (Rasgrf2-2A-dCre; Camk2a-tTA; Ai93) mice from the Allen Institute (10–16 weeks old at the time of surgery).

  For studies of neocortical hemodynamics, we used male mice (10-12 weeks old) that were either of the C57BL/6J strain (Jackson Laboratory) or PV-Cre/Ai14 mice, which express the red fluorophore tdTomato in parvalbumin interneurons.

- Wild animals: We did not use any wild animals.

- Field-collected samples: We did not use any field-collected samples.

- Ethics oversight: The Stanford University Administrative Panel on Laboratory Animal Care approved all procedures using animals.

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