Supplementary Figure 1

Comprehensive opto-mechanical design of the dual-axis microscope.

(a) The complete microscope is shown to scale. The laser beam is depicted in light red.
(b) A close-up view of the front of the microscope showing the opto-mechanical components of the articulated imaging arms.

(c) A close-up view of the top arm (without the miniature mirror) showing the two rotational actuators and three translational actuators embedded in the distal part of the arm.

(d) Each microscope objective lens (0.35 NA) is optically coupled to an individual microendoscope. The microendoscope is a doublet combining a GRIN relay lens and a GRIN micro-objective lens.
Supplementary Figure 2

Simultaneously acquired images of hippocampal area CA1 and the frontal association area (FrA) in an anesthetized mouse expressing the red fluorophore tdTomato in parvalbumin interneurons.

Scale bar is 100 μm and applies to both panels.
Supplementary Figure 3

Computational studies show that an axial resolution of 10 µm leads to minuscule cross talk from out-of-focus plane cell bodies in neurons’ digitized records of Ca\textsuperscript{2+} dynamics.

(a) Using pairs of actual two-photon fluorescence traces of Ca\textsuperscript{2+} activity (traces in top two rows), we calculated levels of cross talk between pairs of layer 2/3 mouse visual cortical neurons hypothetically positioned at different axial separations. Both cells in each pair had identical lateral coordinates. An example cell pair and traces for three example axial separations (12 µm, 14 µm and 20 µm) between the two cells’ centroids are shown (traces in bottom three rows). However, overall we calculated cross talk levels for a total of 132 cell pairs, whose axial separations were varied over a range of hypothetical distance values. From actual two-photon image stacks, we determined that mouse visual cortical neurons have cell body diameters of 12.09 ± 0.05 µm (mean ± s.e.m.; N = 339 layer 2/3 pyramidal cells), in close agreement with prior measurements in this cell type\textsuperscript{24}. We thus modeled each cell as a 12-µm-diameter homogeneous sphere, and used the three-dimensional optical resolution measurements from the poorer-performing arm of the dual-axis microscope to estimate the expected cross talk between pairs of cells at various axial separations. After applying the event detection algorithm\textsuperscript{25} (red asterisks mark detected Ca\textsuperscript{2+} transients), very few instances of fluorescence cross talk ever reached the digitized event traces (black vertical arrows), which were the traces used for all computations of neuronal coding properties.

(b) Computed percentage of false positive events due to cross talk, as a function of the axial separation between the two cells in a pair. Error bars indicate s.e.m. values, computed over 132 cell pairs per data point.
Empirically determined, cumulative distribution of the axial separations between layer 2/3 visual cortical pyramidal cells and their closest neighbors in the axial dimension. By using two-photon microscopy in live mice, we measured the anatomic coordinates of 339 pyramidal neurons in layer 2/3 of mouse visual cortex. For each neuron, we identified its closest neighbor in the axial dimension that could plausibly be a source of cross talk, given the 1.0 µm lateral resolution of the dual-axis microscope. We identified this closest axial neighbor as the nearest cell whose lateral (x,y) centroid coordinates were <3 µm away from the lateral centroid of the first cell.

Using the empirically determined distribution of axial separations between cell pairs (panel c) and the computed level of cross talk as function of axial separation (panel b), we computed the statistical distribution of expected cross talk levels across all 339 cells examined. Overall, >97% of neuronal cell bodies had 0% cross talk in the digitized rasters of action potentials detected in the ΔF/F traces, and 98.5% of neurons had <5% false positive spikes due to cross talk.
Experimental assessment of cross-talk from out-of-focal plane neural elements revealed no statistically significant cross-talk in neurons’ digitized records of Ca$^{2+}$ dynamics.

To directly assess cross talk levels from out-of-focal plane neural elements (somata, dendrites or axons) in behaving mice, we acquired Ca$^{2+}$ activity traces from layer 2/3 visual cortical pyramidal cells at two different optical resolutions simultaneously. To do this, we built a...
two-photon imaging system in which an electro-optical modulator (EOM) toggled the laser beam between two different optical paths that provided different optical resolutions.

(a) Schematic of the optical apparatus. We polarized the beam from an infrared ultrashort-pulsed Ti:Sapphire laser by using a polarizing beam splitter (PBSC). A rotatable half-wave plate (\( \lambda/2 \)) provided a means of power control; a beam block (BB) absorbed the unused power. We electronically controlled the EOM such that on every successive image frame (10 Hz frame rate) the beam alternated between the two optical pathways. In pathway 1, the laser beam was magnified so as to provide higher optical resolution (FWHM axial resolution: 4.0 ± 0.1 \( \mu \)m) in the specimen plane. In pathway 2, the beam was de-magnified so as to provide a lower resolution (FWHM axial resolution: 10.4 ± 0.4 \( \mu \)m) that conservatively matched the 9.8 ± 0.2 \( \mu \)m axial resolution of the dual-axis microscope’s poorer-performing arm. The two beam pathways were re-combined within another polarizing beam splitter (PBSC) and co-aligned as they entered the back aperture of a 0.95 NA microscope objective. This arrangement allowed two temporally interleaved time-traces of Ca\(^{2+} \) activity to be acquired from the very same neurons using a single photomultiplier tube (PMT) to detect fluorescence.

Inset: Successive image frames acquired in a live mouse using the higher- and lower-resolution beam pathways.

(b) Temporally interleaved Ca\(^{2+} \) activity traces acquired at the two different resolutions, from two example cell bodies (top two traces) and two example dendritic processes (bottom two traces). The fluorescence traces acquired at the higher- and lower-resolutions appear almost identical. There is no discernible evidence of any neural sources that are present in the lower-resolution traces but absent in the higher-resolution traces.

(c) Statistical comparisons between even-numbered and odd-numbered frames in a Ca\(^{2+} \) imaging video taken exclusively at the higher resolution (i.e. with the EOM directing the beam only to pathway 1) yielded baseline estimates (cyan data) of the statistical uncertainties in the event detection process for both neural cell bodies and dendrites. In this baseline control data, the sets of detected Ca\(^{2+} \) events that we extracted from the even- and odd-numbered image frames differed by 8.6 ± 1.9% (mean ± s.e.m.) of all Ca\(^{2+} \) events occurring in cell bodies (\( N = 19 \) cell bodies) and by 12.7 ± 3.0% in dendrites (\( N = 23 \) dendrites). When we toggled successive image frames between the higher-resolution and lower-resolution beam pathways, the percentage differences between the sets of detected Ca\(^{2+} \) events in the two data streams (red data) were 6.1 ± 1.7% for cells bodies and 15.7 ± 4.3% for dendrites (\( N = 20 \) cell bodies and \( N = 14 \) dendrites). These percentages of discrepant events between the higher- and lower-resolution data streams were statistically indistinguishable from those when comparing even- and odd-numbered frames of the baseline datasets acquired using only the higher resolution pathway (Wilcoxon rank-sum test; somas: \( P = 0.21 \); dendrites: \( P = 0.46 \); \( N = 20 \) cell bodies and \( N = 14 \) dendrites). Given that the neurons’ measured cell body diameter was 12.09 ± 0.05 \( \mu \)m (mean ± s.e.m; \( N = 339 \) layer 2/3 pyramidal cells), it is extremely unlikely that any further improvements in axial resolution to values finer than 4 \( \mu \)m would significantly change these assessments of cross talk.
Many dendritic processes yielded their own distinct activity patterns as recorded using the dual-axis microscope in awake behaving mice.

(a) Grayscale images are time-averages over 5 min of movie data, acquired by simultaneous Ca$^{2+}$-imaging of visual areas V1 and LM in a head-restrained mouse at liberty to walk or run on a trackball.

(b) Neuronal cell processes that displayed Ca$^{2+}$ activity, as extracted computationally by an established approach to sorting cellular Ca$^{2+}$ signals$^{15}$, are depicted in color. Dendrites marked with numbers have their corresponding time traces shown in panel c.

(c) 20 example traces of Ca$^{2+}$ dynamics in neuronal processes, divided equally between V1 and LM. Blue shaded epochs denote periods of mouse locomotion. Numbers on the traces indicate the corresponding numbered dendrites shown in panel b.

Scale bars in a are 100 μm and also apply to b.
Cell bodies and neuronal processes in both V1 and LM show orientation tuning in their Ca\textsuperscript{2+} transient responses to moving grating visual stimuli.

Gray traces represent the Ca\textsuperscript{2+} dynamics imaged on single trials in cell bodies (top two rows) and neuronal processes (bottom two rows), for each of four orthogonal directions of grating movement (each denoted with a distinct color shading). Red traces depict mean responses averaged over 10 presentations.

Scale bars are 10 μm and 30 μm for images of cell bodies and neuronal processes, respectively.