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Random-access scanning microscopy for 3D imaging in awake behaving animals

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Abstract

Understanding how neural circuits process information requires rapid measurements from identified neurons distributed in 3D space. Here we describe an acousto-optic lens two-photon microscope that performs high-speed focussing and line-scanning within a volume spanning hundreds of micrometres. We demonstrate its random access functionality by selectively imaging cerebellar interneurons sparsely distributed in 3D and by simultaneously recording from the soma, proximal and distal dendrites of neocortical pyramidal cells in behaving mice.

Keywords

Calcium imaging; two-photon; acousto-optic lens; random-access; synapse; neuron; network; circuit

Understanding information processing in the brain requires the measurement of signals as they rapidly flow through neuronal circuits, deep within scattering tissue. However, circuits are often arranged in layers, neuronal subtypes are sparsely distributed, and dendrites and axons radiate and branch in 3D space. Since neuronal compartments of interest often occupy a small fraction of total tissue volume, considerably higher temporal resolution could be achieved if regions of interest (ROIs) were selectively imaged rather than the whole volume. Although conventional resonant galvanometer two-photon laser scanners can image rapidly¹ and achieve fast focussing with electrically tuneable² or ultrasound lenses³ or with a pair of

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Data Availability Statement

Data are available upon request.

Software Availability

The SilverLab LabVIEW Imaging Software is available on GitHub: <https://github.com/SilverLabUCL/SilverLab-Microscope-Software>.

Author Contributions

R.A.S. supervised the project; H.R. and C.B. performed *in vivo* experiments; K.M.N.S.N. analysed the data; V.G. G.K. and K.M.N.S.N. developed microscope control and acquisition systems; K.M.N.S.N., T.K., V.G. and G.J.E. wrote the LabVIEW imaging software; G.J.E. and P.A.K. developed the AOL model and performed simulations; P.A.K., K.M.N.S.N. and R.A.S. designed the microscope; R.A.S., K.M.N.S.N. and P.A.K. wrote the manuscript.

Competing financial interests

The authors declare competing financial interests: details accompany the full text HTML version of the paper at <http://www.nature.com/naturemethods/>. Patents on the AOL technology have been filed (WO/2008/032061, WO/2011/131933).

matched objective lenses and a small mirror⁴, their inertia limits the speed at which they can jump between ROIs. This makes it difficult to monitor sparsely distributed neuronal activity in 3D with conventional imaging methods.

Acousto-optic lens (AOL) microscopes^{5–7} can perform inertia-free focussing enabling acquisition of 3D Random-Access Multi-Photon (3D-RAMP) point measurements within an imaging volume at 35–50 kHz^{7–10}. However, the major technical limitation of acousto-opticdeflector (AOD)-based scanning, including conventional AOLs, is that they cannot perform full-frame continuous line-scanning away from the natural focal plane of the objective^{6,11,12} (Supplementary note 1,2). This is problematic for *in vivo* imaging because acquiring Z-stacks is slow using 3D-RAMP voxel-by-voxel pointing⁷ or mini-scans (consisting of a few voxels)⁹ to form each image, due to their low duty cycle compared to continuous line-scans (Supplementary Fig. 1a). Furthermore, brain movement compromises 3D-RAMP pointing, because points of interest shift away from the predefined points where the laser is sequentially focussed. To overcome these limitations, we have developed a compact AOL two-photon microscope that can perform 3D Random-Access Pointing and continuous line-Scanning (3D-RAPS) over the entire imaging volume. This enables high-speed volumetric imaging and a range of new random access 3D imaging modes for monitoring sparsely distributed neuronal activity in awake animals (Fig 1a).

Three technical developments were required for AOL-based random access line-scanning. Firstly, custom-designed, thin on-axis AOD crystals, coupled with elliptically polarised light, improved uniformity of diffraction efficiency with deflection angle (Supplementary Fig. 2a) and AOL light transmission efficiency (from ~15% to ~25%) over our 1st generation design. Secondly, high-speed line-scanning generated with our custom-designed field programmable gate array (FPGA)-based AOL control system (Fig 1a; Supplementary Fig. 1b; Methods) was key in enabling more uniform light transmission over larger scan angles when focussing away from the natural focal plane, as predicted by our model of a spherical AOL¹³ (Fig. 1b). This occurs because fast scanning reduces the time the AOL is required to focus, allowing more acoustic bandwidth for beam deflection (Supplementary Note 2). Thirdly, a high bandwidth FPGA-based acquisition system was synchronised with the AOL control system to acquire red and green fluorescence signals during fast line-scans (Methods). These developments resulted in a compact AOL 3D two-photon laser scanner with relatively uniform transmission efficiency across the focussing range (Supplementary Fig. 2b). As predicted, the focussing range over which full 512 voxel XY scanning could be performed depended on line-scan speed (Supplementary Fig. 1c). Since optical aberrations associated with remote focussing and diffractive optics increase with imaging volume size (Supplementary Fig. 3)⁶, we restricted high resolution imaging to 250x250x250 μm . This volume could be imaged using full-frame constant velocity line-scans (512x512 or 1024x1024 voxels) with a 50 ns/voxel dwell time (i.e. pixel integration time; Supplementary Fig. 2c; Supplementary Video 1), which represents a >255-fold increase in voxel acquisition rate compared to the fastest AOL 3D-RAMP pointing and is comparable to 2D resonant galvanometers and 2D AOD scanners (Supplementary Table 1).

To test the *in vivo* performance of our AOL microscope we imaged cerebellum and visual cortex in awake head-fixed mice on a cylindrical treadmill¹⁴. The objective lens was fixed

and focussing and line-scanning performed exclusively by the AOL. The spatial resolution of AOL Z-stack imaging was sufficient to resolve fine structures of L2/3 pyramidal cells expressing the activity-independent protein tdTomato (Supplementary Video 1). Spines were resolvable over the full 250 μm focus range and across the XY field-of-view (FOV; Supplementary Fig. 4). In the cerebellum, this focussing range enabled imaging across all three layers of the cortex (Fig. 1c; Supplementary Fig 5a). For the fastest full-frame line-scan speed (20 kHz) the AOL could image at 39 Hz at 512x512 voxel resolution. Since the AOL can focus to any location within the imaging volume and start the next line-scan within 24.5 μs , full resolution Z-stacks could be acquired rapidly (Supplementary Video 2).

To examine neuronal activity in 3D we expressed the genetically encoded calcium indicator GCaMP6f15 in the cerebellar cortex and performed multi-plane imaging. Superficial and deep molecular layer interneurons are visible in the upper two images (49 μm and 73 μm from pia), while superficial and deep Golgi cells were recorded in upper and lower regions of the granule cell layer (GCL; at 85 μm and 102 μm from pia) in the four 256x256 voxel images recorded near-simultaneously at 19.5 Hz (Fig. 1d; Supplementary Fig 5b; Supplementary Video 3). Following *post hoc* movement correction of images (Methods), analysis of the fluorescence revealed calcium signals related to locomotion in these inhibitory interneurons (Fig. 1d). These results demonstrate that our AOL microscope design can perform high-speed Z-stacks and multi-plane imaging, overcoming major limitations of previous AOL designs and extending methods for imaging neuronal activity across cortical layers^{2,16}.

Inhibitory interneurons typically occur at a lower density than excitatory neurons and are sparsely distributed in space. This makes monitoring activity with single or multi-plane imaging inefficient, because the probability of a soma falling within the imaging plane is small (Supplementary Fig. 6a,b). To address this we implemented random access patch scanning (Fig 1a), enabling the user to define imaging patches intersecting each ROI. Such selective ROI imaging allows more sparsely distributed cells to be monitored at high temporal resolution within the imaging volume (Supplementary Fig. 6a-c). The smaller number of voxels per line used for patches than for full line-scans enabled faster imaging (Supplementary Fig. 1d) and a wider range of dwell times (Supplementary Fig. 2c,d). Random access patch imaging of cerebellar interneurons at 50 Hz (Supplementary Video 4) revealed regular, discrete signalling events during both stationary periods and during locomotion (Supplementary Fig. 7). In contrast, patch imaging of L2/3 pyramidal cells co-expressing GCaMP6f and tdTomato (Supplementary Video 5) revealed sparse activity that was not clearly related to locomotion (Fig. 2). Similar results were obtained across a range of dwell times in animals expressing only GCaMP6f (Supplementary Fig. 8). By using a montage of patches for *post hoc* movement correction, we ensured spatial information was present even when GCaMP6f fluorescence dropped to low levels in some cells (Supplementary Video 5), enabling accurate determination of masks for functional analysis (Supplementary Fig. 9). Key advantages of patch scanning over 3D-RAMP point measurements, which can also be performed with our compact AOL design^{6,8}, are that ROIs can be imaged with a higher duty cycle (Supplementary Fig. 1a) and that spatial information within patches allows *post hoc* correction of brain movement, enabling reliable recordings from awake animals.

Understanding how dendritic activity relates to neuronal output requires measurement from dendrites and soma or axon. However, apical dendrites are often orientated axially towards the pia in *in vivo* preparations, making it difficult to rapidly monitor dendritic and somatic activity simultaneously with current imaging technologies. To examine whether AOL-based patch scanning could solve this problem, we selectively imaged the soma, main dendritic trunk and the tuft nexus of a L5 pyramidal cell expressing GCaMP6f and tdTomato in an awake animal (Fig. 3a). Despite the 3 ROIs spanning an extended focal range of 297 μm , with the deepest patch over 400 μm from the pia, robust functional signals could be recorded from each location simultaneously at >200 Hz (Fig. 3b,c). Random access patch scanning can also be used to build up sub-volumes (Fig. 1a). To examine whether this could provide a more complete picture of dendritic activity we imaged three sub-volumes encompassing the soma, proximal dendrite and distal dendrite of a layer 2/3 pyramidal cell at 27.9 Hz (Fig. 3d,e). This revealed $[\text{Ca}^{2+}]$ transients in the dendrites and soma with similar activity patterns (Fig. 3f). These results demonstrate that sparsely distributed neuronal structures can be selectively imaged with AOL-based random access patch and sub-volume imaging, substantially faster than with whole volume raster scanning.

Our 3D AOL-scanner extends the functionality of current galvanometer and axial scanning-based 3D two-photon imaging technologies^{2–4,16,17} (Supplementary Table 1; Supplementary Note 3) and overcomes a major limitation of previous AOL designs by providing ultra-fast inertia-free focussing and continuous, constant-velocity XY line-scanning in any focal plane. The compact dimensions (20x20x30 cm) of our AOL design and location on the optical bench, mean that it can be added to custom-built (Supplementary Fig. 2e) or commercial two-photon microscopes⁸. Moreover, recent studies indicate that spatial resolution could be further improved with AOL-based wavefront shaping^{18,19}. AOL scanning could also be used in conjunction with fast voltage indicators, photolabile compounds and optogenetic transducers for high-speed 3D photostimulation²⁰. Our results show that by enabling 3D-RAPS our AOL microscope design can selectively image sparsely distributed populations of inhibitory interneurons without wasting time imaging ‘dead space’ in-between. Moreover, its ability to monitor dendritic and somatic activity simultaneously, over large depth ranges at high-speed, provides a new tool for studying dendritic integration in awake behaving animals.

Online Methods

Compact Acousto-Optic Lens 3D two-photon microscope

The optical layout of the 3D Acousto-Optic Lens (AOL) two-photon microscope (Fig. 1a) consisted of a Femtosecond laser (2 W at 920 nm; Chameleon Ultra II, Coherent Inc.), a custom designed prism-based pre-chirper that introduced -29000 fs² group velocity dispersion (APE GmbH, Berlin), a Pockels cell (Model 350-80LA, Conoptics) and a beam expander that filled the 15 mm aperture of the AOL⁶. The second generation compact configuration AOL reported here consists of two orthogonally arranged pairs of on-axis TeO₂ AODs (Gooch and Housego). AODs were interleaved with quarter wave plates and polarizers to couple the beam into subsequent AODs and also to block the unwanted zero order beam. Acoustic transducers on the first AOD of each pair were wider than the second

AOD6 to maximize diffraction efficiency and the input angle range, respectively. The compact AOL assembly was contained in a 20x20x30 cm box located on the optical table and coupled to a custom-built microscope via a 4f relay (Supplementary Fig. 2e). Compact AOLs can also be added to commercially available two-photon microscopes with a prechirper by centring the position of the galvanometer mirrors⁸.

The microscope consisted of an in-house optical arrangement mounted on top of a SliceScope (Scientifica, UK) with a tube lens, arranged to under-fill a water immersion objective (Olympus XLUMPlanFLN 20X, NA1.0), with an excitation numerical aperture of 0.6 - 0.7, giving a spatial resolution as quantified in Supplementary Fig. 3. The two-channel detection system consisted of a dichroic mirror (575dcxr, Chroma Inc.) and emission filters (HQ 525/70m-2P and HQ 630/100m-2P). Green light was detected with GaAsP PMT (H7422, Hamamatsu, Japan) and red light with either a standard PMT (R9880U-20, Hamamatsu, Japan) or a GaAsP (H7422, Hamamatsu, Japan). Output signals from the PMTs were amplified using 200 MHz pre-amplifiers (Series DHPVA 100/200 MHz, FEMTO). The mechanical focus and stage were both motorised (Scientifica Model-MMBP) and computer controlled. The animals were head fixed using two custom built retractable arms that mounted onto the animal head plate. The treadmill consisted of a Styrofoam cylinder and locomotion was detected with a rotary encoder (RI58, Hengstler, Germany).

The custom designed FPGA AOL control system (Supplementary Fig. 1b) consisted of a Xilinx ML506 card and a Texas Instruments DAC card (DAC5672EVM)¹⁹. Commands to control the loading and execution of the frequency records for 3D AOL random-access raster scanning, patch imaging, multi-volume and pointing were generated by the PC and encoded as RAW Ethernet packets, before being transmitted to the AOL controller via a Gigabit Ethernet interface. The AOL control FPGA used an on-chip, direct digital synthesiser to generate the specified acoustic frequency chirps and these were executed upon receiving a start trigger from data acquisition system. The synthesised digital waveforms were converted into analogue signals by Digital-to-Analog Converters (Texas Instruments, DAC5672EVM) and amplified by 4 RF amplifiers before being fed into the AODs. The FPGA AOL control system design and compiled firmware are available for non-commercial research on request.

The acquisition system consisted of a high speed ADC (800 MHz, dual channel, NI-5772) together with an FPGA (NI FlexRIO – 7966R) that enabled forty-fold oversampling for dwell times of 50 ns. The DAQ FPGA acted as the master, synchronising the AOL acoustic frequency ramps and the data acquisition, allowing dwell times of 50–500 ns for scanning and 50-8000 ns for pointing. Signals were down sampled on the DAQ FPGA by integrating over each voxel dwell time before sending frames to the host PC via the National Instruments PXIe interface. Voxel integration was not synchronised to the 80 MHz laser pluses. Peripherals were controlled using a PXI 6733 card.

The data acquisition software running on the NI FPGA and the user interface (UI) running on Windows PC were developed in LabVIEW and information was exchanged via NI PXIe interface. The software architecture is modular and can instruct the AOL-control system to perform a number of imaging modes including continuous full frame raster scanning, fast Z-stacks, random-access sub-volume imaging, random-access patch imaging, 3D RAMP

pointing as well as setting the resolution, field of view, zoom and dwell time. In order to compensate for the effects of tissue scattering, excitation intensity was adjusted as a function of depth using a Pockels cell. Powers used for *in vivo* imaging were typically 20 - 60 mW, depending on imaging depth. The size of patches was chosen so that the object of interest remained within the patch. In practice, this does not necessarily lead to a reduction in temporal resolution as the voxel resolution of the patch can be reduced as the size is expanded. Acquired data was continuously streamed to disk and depending on the type of recording stored as TDMS, TIFF, or AVI files.

***In vivo* functional imaging in awake behaving mice**

All procedures were carried out in accordance with institutional animal welfare guidelines and licensed by the UK Home Office. We transfected neurons in the mouse visual cortex and cerebellar cortex with a genetically encoded calcium indicator GCaMP6f15. Dual sparse labelling in the visual cortex was achieved by co-injecting Cre recombinase (AAV9.CamKII0.4.Cre.SV40, diluted 1:10,000, UPenn Vector Core) with Flex-GCaMP6f (AAV1.Syn.Flex. GCaMP6f.WPRE.SV40, diluted 1:2) in Ai9 cre-reporter tdTomato15 transgenic adult mice (tdTomato mice (B6;129S6-*Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J}*). The somata of cells expressing tdTomato and GCaMP6f appear filled (e.g. Fig. 2), due to the spectral overlap between tdTomato and GCaMP6f. In contrast, when GCaMP6f is expressed alone, somata had a hollow appearance (Supplementary Fig. 8). For these animals, Cre recombinase (AAV9.CamKII0.4.Cre.SV40, diluted 1:5,000) and Flex-GCaMP6f (AAV1.Syn.Flex. GCaMP6f.WPRE.SV40, diluted 1:2) were co-injected in C57Bl/6 mice. For the cerebellar imaging, either the GAD65-GFP transgenic mouse strain was used or a mGluR2-Cre-IRES-eGFP mouse strain (B6;FVB-Tg(Grm2-cre,-EGFP)631Lfr/Mmuc). For functional measurements, mice were injected with the virus construct AAV1.SynFlex.GCaMP6f.WPRE.SV40 (UPenn Vector Core) into the cerebellar cortex. Stereotaxic virus injections were performed on mice of both sexes, (three male, two female) between P30-P50 for the experiments on visual cortex and older than P60 for experiments in cerebellum. Animals were anaesthetised with ketamine/xylazine, and were slowly injected with a 10-100 nL of suspended virus at a depth of 100-400 μ m. A cranial window was made by placing a 4 mm coverslip gently over the craniotomy and implanting a head plate onto the skull with dental cement. Mice were familiarised with the setup for 1-3 days and then imaged from 4 weeks after viral injection. Mice were free to stand or run on the cylindrical Styrofoam wheel during imaging.

Data format and analysis

Raw imaging data was stored as TIFF images for each time series of the plane/patch. TIFF images were corrected for brain motion using the sequential image analysis software suite (SIMA)21. For patch imaging, movement was either corrected patch-by-patch or using a montage of all the patches. The latter approach was particularly effective for imaging ROIs that were sparsely active and in mice expressing only GCaMP6f (Supplementary Video 6). Functional signals were extracted from images by generating masks of cellular structures (Supplementary Figure 9) using a thresholding algorithm or the thresholding and particle analysis (ImageJ). The average of the intensity of voxels across the mask was calculated for

each frame and F/F calculated. The timing of each data point was calculated at the centre of the ROI.

ImageJ and neuTube (neuro.org/content/early/2015/01/02/ENEURO.0049-14.2014) were used for 3D rendering and to adjust brightness and contrast of the images for display purposes. No deconvolution or nonlinear scaling such as gamma correction was used on the data for fluorescence traces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Svoboda K, Yasuda R. Principles of two-photon excitation microscopy and its applications to neuroscience. *Neuron*. 2006; 50:823–839. DOI: 10.1016/j.neuron.2006.05.019 [PubMed: 16772166]
2. Grewe BF, Voigt FF, van 't Hoff M, Helmchen F. Fast two-layer two-photon imaging of neuronal cell populations using an electrically tunable lens. *Biomed Opt Express*. 2011; 2:2035–2046. DOI: 10.1364/BOE.2.002035 [PubMed: 21750778]
3. Kong L, et al. Continuous volumetric imaging via an optical phase-locked ultrasound lens. *Nat Methods*. 2015; 12:759–762. DOI: 10.1038/nmeth.3476 [PubMed: 26167641]
4. Botcherby EJ, et al. Aberration-free three-dimensional multiphoton imaging of neuronal activity at kHz rates. *Proc Natl Acad Sci U S A*. 2012; 109:2919–2924. DOI: 10.1073/pnas.1111662109 [PubMed: 22315405]
5. Kaplan A, Friedman N, Davidson N. Acousto-optic lens with very fast focus scanning. *Opt Lett*. 2001; 26:1078–1080. [PubMed: 18049525]
6. Kirkby PA, Srinivas Nadella KM, Silver RA. A compact Acousto-Optic Lens for 2D and 3D femtosecond based 2-photon microscopy. *Opt Express*. 2010; 18:13721–13745. DOI: 10.1364/OE.18.013720 [PubMed: 20588506]
7. Reddy GD, Saggau P. Fast three-dimensional laser scanning scheme using acousto-optic deflectors. *J Biomed Opt*. 2005; 10:064038.doi: 10.1117/1.2141504 [PubMed: 16409103]
8. Fernandez-Alfonso T, et al. Monitoring synaptic and neuronal activity in 3D with synthetic and genetic indicators using a compact acousto-optic lens two-photon microscope. *J Neurosci Methods*. 2014; 222:69–81. DOI: 10.1016/j.jneumeth.2013.10.021 [PubMed: 24200507]
9. Katona G, et al. Fast two-photon in vivo imaging with three-dimensional random-access scanning in large tissue volumes. *Nat Methods*. 2012; 9:201–208. DOI: 10.1038/nmeth.1851 [PubMed: 22231641]
10. Froudarakis E, et al. Population code in mouse V1 facilitates readout of natural scenes through increased sparseness. *Nat Neurosci*. 2014; 17:851–857. DOI: 10.1038/nn.3707 [PubMed: 24747577]

11. Roorda RD, Hohl TM, Toledo-Crow R, Miesenbock G. Video-rate nonlinear microscopy of neuronal membrane dynamics with genetically encoded probes. *J Neurophysiol.* 2004; 92:609–621. DOI: 10.1152/jn.00087.2004 [PubMed: 14999051]
12. Chen X, et al. LOTOS-based two-photon calcium imaging of dendritic spines in vivo. *Nat Protoc.* 2012; 7:1818–1829. DOI: 10.1038/nprot.2012.106 [PubMed: 22976353]
13. Evans GJ, Kirkby PA, Naga Srinivas Nadella KM, Marin B, Angus Silver R. Development and application of a ray-based model of light propagation through a spherical acousto-optic lens. *Opt Express.* 2015; 23:23493–23510. DOI: 10.1364/OE.23.023493 [PubMed: 26368449]
14. Ozden I, Dombeck DA, Hoogland TM, Tank DW, Wang SS. Widespread state-dependent shifts in cerebellar activity in locomoting mice. *PLoS One.* 2012; 7:e42650.doi: 10.1371/journal.pone.0042650 [PubMed: 22880068]
15. Chen TW, et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature.* 2013; 499:295–300. DOI: 10.1038/nature12354 [PubMed: 23868258]
16. Yang W, Miller K, Carrillo-Reid L, Pnevmatikakis E, Paninski L, Yuste R, Peterka DS. Simultaneous multi-plane imaging of neural circuits. *Neuron.* 2016; 89:269–284. [PubMed: 26774159]
17. Cheng A, Goncalves JT, Golshani P, Arisaka K, Portera-Cailliau C. Simultaneous two-photon calcium imaging at different depths with spatiotemporal multiplexing. *Nat Methods.* 2011; 8:139–142. DOI: 10.1038/nmeth.1552 [PubMed: 21217749]
18. Akemann W, et al. Fast spatial beam shaping by acousto-optic diffraction for 3D non-linear microscopy. *Opt Express.* 2015; 23:28191–28205. DOI: 10.1364/OE.23.028191 [PubMed: 26561090]
19. Konstantinou G, et al. Dynamic wavefront shaping with an acousto-optic lens for laser scanning microscopy. *Optics Express.* 2016; 24:6283–6299. DOI: 10.1364/OE.24.006283 [PubMed: 27136821]
20. Emiliani V, Cohen AE, Deisseroth K, Hausser M. All-Optical Interrogation of Neural Circuits. *J Neurosci.* 2015; 35:13917–13926. DOI: 10.1523/JNEUROSCI.2916-15.2015 [PubMed: 26468193]
21. Kaifosh P, Zaremba JD, Danielson NB, Losonczy A. SIMA: Python software for analysis of dynamic fluorescence imaging data. *Front Neuroinform.* 2014; 8:80.doi: 10.3389/fninf.2014.00080 [PubMed: 25295002]

Figure 2. Random-access patch imaging of neurons in layer 2/3 of primary visual cortex in an awake behaving mouse.

a) Maximum intensity projection of red channel for cells in layer 2/3 of visual cortex sparsely expressing td-Tomato and GCaMP6f. **b)** Location of 14 X-Y patches within the imaging volume, distributed between 74 µm to 163 µm below the pia. **c)** Averaged images of cellular structures (green channel) scanned near-simultaneously at 50 ns/voxel in the 14 patches (51 x 50 voxels, 22 µm x 21.5 µm) after *post hoc* correction for brain movement. Traces to the right show $\Delta F/F$ responses extracted from each patch (52.8 Hz patch cycle rate, i.e. $1/(\text{time to scan all patches})$). Grey trace at the bottom shows the speed of the animal on

the cylindrical treadmill. Blue-grey shading indicates periods of locomotion. Scale bars as defined in figure 1 legend.

Figure 3. Simultaneous dendritic and somatic imaging of pyramidal neurons in the visual cortex of awake mice.

a) Partially reconstructed image of a layer 5 neuron within the imaging volume (red box) together with the location of 3 user-selected patches intersecting the soma, apical dendrite and the nexus of the distal dendritic tuft. **b)** Averaged images of the soma and dendritic structures scanned near-simultaneously at 100 ns/voxel in the 3 patches (67 x 51 voxels, ~31 μm x ~23 μm) after *post hoc* correction for brain movement, together with mask outlines (green lines) for extraction of functional data. The acquisition rate was 209 Hz for the 3 patch cycle. Traces to the right show $\Delta F/F$ responses extracted from each colour coded patch while the animal was stationary. Dashed black box indicates measurements from the soma and dendrites that were confirmed to be from the same reconstructed cell, while the two other dendrites could not be unequivocally confirmed from the Z-stack image. Grey trace at the bottom shows the speed of the animal on the cylindrical treadmill. **c)** As for **b** but during locomotion. **d)** Two planes showing the somatic and dendritic sections of cells in layer 2/3 visual cortex sparsely expressing td-Tomato and GCaMP6f. **e)** Location of 3 sub-volumes within the imaging volume together with a partial reconstruction of an imaged L2/3 neuron that includes the soma, proximal dendrite and distal dendrite. Each sub-volume consists of 5-6 planes (224 x 59 voxels, 96 μm x 25 μm) 4 μm apart. **f)** Images of different regions of the pyramidal cell from the planes making up each sub-volume after *post hoc* movement correction. Traces show $\Delta F/F$ responses extracted from the cellular component present in each plane, where red, green and blue indicate soma, proximal dendrite and distal dendrite, respectively. Data were acquired at 50 ns/voxel with a sampling rate of 27.9 Hz for the 3 volume cycle. Grey trace at the bottom shows the speed of locomotion of the animal on the

cylindrical wheel. Blue-grey shading indicates periods of locomotion. Scale bars as defined in figure 1 legend.