

The Cousa objective: a long working distance air objective for multiphoton imaging *in vivo*

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Abstract

Two-photon microscopy can resolve fluorescence dynamics deep in scattering tissue, but applying this technique *in vivo* is limited by short working distance water-immersion objectives. Here we present an ultra long working distance (20 mm) air objective called the Cousa objective. It is optimized for performance across multiphoton imaging wavelengths, offers a >4 mm² FOV with submicron lateral resolution, and is compatible with commonly used multiphoton imaging systems. We share the full optical prescription, along with data on real world performance including *in vivo* calcium imaging in a range of species and approaches.

1. Introduction

Two-photon microscopy of *in vivo* neuronal activity in larger animals, such as monkeys and ferrets, has been challenging due to the mechanical limitations of conventional multiphoton objectives [1–5]. Commercially available microscope objectives with good multiphoton performance often have short working distances (1–10 mm) and/or require water immersion. The short working distance, coupled with the geometry of the objective tip, requires excessive tissue removal and a large cranial window. Such large windows cannot be easily centered over regions of interest, and can exacerbate immune responses and degrade tissue clarity, ultimately limiting the imaging depth and the duration of longitudinal imaging. Water immersion can require awkward reservoirs or the use of gels that can lack appropriate refractive indices and harbor air bubbles that degrade image quality.

To address these issues, we designed an air immersion objective with a working distance of 20 mm, named the Cousa objective. The optics are designed to minimize aberrations for

49 two-photon imaging. The objective has a numerical aperture (NA) of 0.50, which supports
50 submicron lateral resolution imaging. The objective provides diffraction-limited performance
51 over a $>4 \text{ mm}^2$ field-of-view (FOV). The Cousa objective was designed to be compatible with
52 commercial two-photon imaging systems, with standard threading and an entrance pupil of $\varnothing 20$
53 mm. The Cousa objective enables a range of experiments in various species, using commercial
54 off-the-shelf imaging systems.

55 2. Design

56 2.1. Specifications and constraints

57 The design specifications of the 20 mm long working distance objective (**Table 1**) are set
58 and balanced primarily around three factors: (i) geometric parameters to facilitate use in
59 animal imaging applications, (ii) optimization for two-photon imaging across a large FOV
60 with sub-cellular resolving power, and (iii) compatibility with commercial two-photon imaging
61 systems.

62 First, a top priority of the design, its *raison d'être*, is compatibility with animal experiments
63 and the associated instrumentation. Two-photon imaging in neuroscience is often performed
64 through a cranial window. The objective is then positioned above the window, at a distance
65 determined by the working distance of the objective. This arrangement can pose constraints
66 on imaging. For example, ferrets and other animals have skulls that are $> 1 \text{ mm}$ thick with
67 a significant gap between the skull and dura mater. In these cases, cranial windows must be
68 enlarged to accommodate standard two-photon objectives, due to their short working distances
69 and tip geometries. Such large imaging windows create challenges for window positioning,
70 imaging quality, and long-term maintenance. For additional examples, even in smaller animals
71 such as mice, short working distances prevent the insertion of auxiliary optics between the
72 objective and sample, and can also prevent imaging in complex preparations such as ventral
73 access to the cochlea.

74 To address these issues, we started with the requirement that the working distance would be
75 long, 20 mm. By using a long working distance design, the objective can remain comfortably
76 outside of an imaging chamber, resulting in fewer mechanical constraints. We also recognized
77 that imaging at angles other than the conventional vertical orientation, in particular in larger
78 animals, can make maintaining water immersion difficult. Thus, for this design we chose to use
79 air immersion. Air immersion entails a larger refractive index mismatch than water immersion
80 designs, so we mitigated the trade-off by incorporating a correction collar that can compensate
81 for aberrations.

82 Second, the lens design was optimized for focusing ultrafast laser pulses centered at wavelengths
83 commonly used in two-photon imaging, including popular genetically encoded calcium indicators
84 like the GCaMP series [6–8]. The optics were designed to offer diffraction-limited performance
85 across a range of wavelengths. We set the NA to be 0.50, corresponding to a diffraction-limited
86 resolution of $0.69 \mu\text{m}$ laterally and $5.84 \mu\text{m}$ axially, which is sufficient to resolve neurons,
87 dendritic spines, and axonal boutons [9, 10].

88 Third, the objective was designed to be compatible with commercial multiphoton imaging
89 systems. Major microscope manufacturers use infinity conjugate (i.e., infinity-corrected) optical
90 designs [11], and thus we adopted the same convention for compatibility. The other main
91 constraints from commercial two-photon imaging systems are the beam diameter at the objective
92 back aperture and the maximal scan angle. These parameters are determined by the scan engine.
93 Many commercial two-photon imaging systems constrain the maximal beam diameter to about
94 20 mm, and the scan angles to about $\pm 3^\circ$. We adopted these commonly attained values as design
95 specifications. In addition, we adopted the M32 x 0.75 thread size, which is used on many
96 multiphoton imaging objectives, and thus adapters are readily available.

97 With these specifications and constraints set (working distance, air immersion, numerical

Working distance*	20 mm
Immersion media*	Air
Numerical aperture*	0.50
Effective focal length*	20 mm
Magnification	10 X (with a 200 mm focal length tube lens)
Correction collar*	0 - 1 mm cover glass thickness
Entrance pupil*	20 mm
Scanning angles*	$\pm 3^\circ$
Field of view (FOV)	2.08 mm
Primary wavelength range*	920 nm \pm 10 nm
Full wavelength range	800 - 1300 nm
Anti-reflective coating	< 0.5% reflected 450-1100 nm (per surface)
Parfocal distance	\sim 90 mm
Mounting threads*	M32 x 0.75
Weight	477 g
Group delay dispersion	~ 4910 fs ² at 920 nm

Table 1. Specifications of the long working distance air objective. Parameters that were used to constrain the initial design are marked with an asterisk (*).

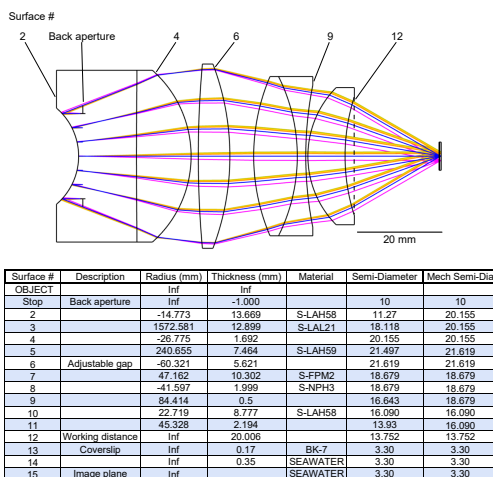


Fig. 1. Lens layout and prescription. The maximal scan field is $\pm 3^\circ$, and the effective focal length is 20 mm. The adjustable air gap is set at the thickness of Surface 6 to implement the function of a correction collar. The working distance, which is 20 mm, is the air space between the objective tip (Surface 12, dashed line) to the surface of the coverslip glass (Surface 13). The optimization routines were set assuming a focal plane ~ 0.35 mm into the brain, as indicated on the prescription (Surface 14).

98 aperture, scan angle, and back aperture diameter), a relatively large FOV remains feasible by
 99 setting the effective focal length of the objective to 20 mm [12]. The design and optimization
 100 process was conducted using optical simulation software (ZEMAX OpticStudio). The merit

101 function prioritized maximizing the working distance while maintaining a diffraction-limited
 102 point spread function and minimizing the wavefront error across the FOV. Parallel efforts were
 103 made to reduce the number of lenses and the thickness of the materials, and thus minimize the
 104 size, weight, and cost of the final design.

105 2.2. Design and model performance

106 The infinity corrected objective consists of six lens elements (**Fig. 1**) with a net group delay
 107 dispersion of $\sim 4910 \text{ fs}^2$ at 920 nm [13]. The working distance (surface 12 to the focal plane at
 108 surface 15) is ~ 20 mm. The position of the back aperture was designed to be very close to the first
 109 element, surface 2. This facilitates alignment in commercial systems, since visual inspection at
 110 the back surface can determine whether the excitation beam remains stationary during scanning.
 111 In the model, the root-mean-square (RMS) wavefront error for 920 ± 10 nm light is less than
 112 0.02λ across the scan angles, which is considerably less than the diffraction limit of 0.072λ (**Fig.**
 113 **2a**). Similarly, the Strehl ratio [14] is over 0.97 across the nominal $\pm 3^\circ$ scan angles (**Fig. 2b**),
 114 exceeding the diffraction limit of 0.8. Thus the performance is diffraction-limited throughout
 115 the designed FOV by a large margin. This margin provides some assurance that performance
 116 will remain diffraction-limited despite real-world imperfections that are incorporated during
 117 fabrication and assembly.

118 The objective has an air gap between surfaces 6 and 7 that is adjusted by a rotating correction
 119 collar. Correction collar adjustments can compensate for a range of cover glass (surface 13)
 120 thicknesses, from 0 to 1.0 mm. The correction collar can also be adjusted to optimize performance
 121 at different excitation wavelengths (**Fig. 2**). Adjusting two free parameters, the precise focal
 122 plane location and the correction collar position, diffraction-limited performance can be extended
 123 to a range of 800-1300 nm for multiphoton excitation at a range of wavelengths (**Fig. 2c, 2d,**
 124 **and Table 2**). Note that the refocusing is applied at a single position for all scan angles, and the
 125 merit function balances performance over the full FOV.

Refocus by adjusting objective-sample air gap (surface 12)*												
Wavelengths (nm)	800	850	900	950	1000	1050	1100	1150	1200	1250	1300	
Air gap at surface 12 (mm)	20.018	20.009	20.006	20.007	20.011	20.018	20.027	20.038	20.05	20.065	20.08	
*Thickness of surface 6 = 5.621 mm; Coverslip thickness = 0.17mm												
Refocus by adjusting objective-sample air gap (surface 12) and correction collar (surface 6)**												
Wavelengths (nm)	800	850	900	950	1000	1050	1100	1150	1200	1250	1300	
Air gap at surface 12 (mm)	20.011	20.006	20.005	20.007	20.012	20.018	20.026	20.035	20.046	20.057	20.07	
Air gap at surface 6 (mm)	5.669	5.643	5.626	5.618	5.615	5.619	5.627	5.639	5.656	5.675	5.699	
**Coverslip thickness = 0.17mm												

Table 2. The nominal thicknesses of air gaps used for different wavelengths. The upper portion of table lists the distance of the objective-to-sample gap (Surface 12) when this single air gap is adjusted for different wavelengths. The lower part of the table shows the thicknesses of the objective-to-sample gap (Surface 12) and the correction collar gap (Surface 6) when both of these two air gaps are adjusted.

126 2.3. Mechanical model and assembly

127 After optical designs were finalized, the mechanical design, lens fabrication, housing manufac-
 128 turing, and objective assembly processes were contracted to an external firm (Special Optics,
 129 Denville, NJ, USA). The objective is 79 mm long and 65 mm wide, and the total weight is 477
 130 grams (**Fig. 3**). The long working distance of the objective relaxes the geometric constraints of
 131 the design, as was our strategy. However, one mechanical constraint remained: the objective
 132 needed to fit within the clearance around the objective mounting threads of commonly used
 133 multiphoton microscopes. A conventional way to load the optics into an objective is to leave
 134 the back open, insert all lenses, and then seal it off. This stacking approach leads to the largest

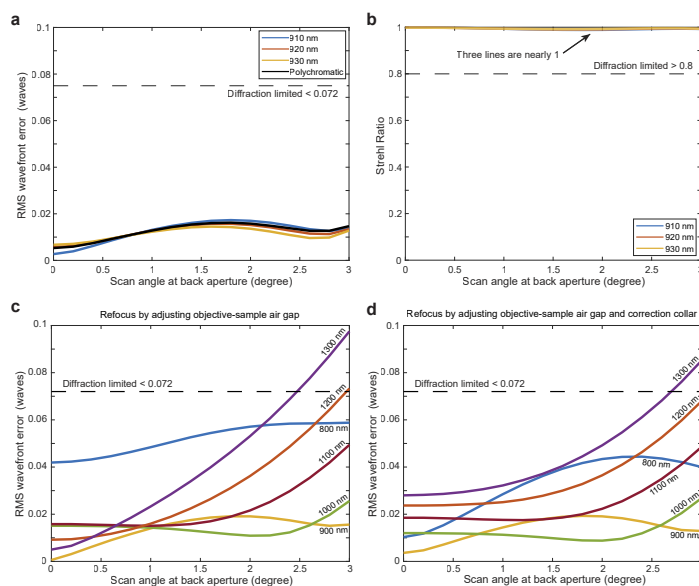


Fig. 2. Performance analysis. The primary optimizations were for 920 ± 10 nm, for two-photon excitation of green fluorescent protein (GFP)-based indicators with ultrafast pulses of light. The optical model predicts (a) low root-mean-squared (RMS) wavefront errors and (b) high Strehl ratios for 910 nm, 920 nm, and 930 nm light across the scan angles of $0 - 3^\circ$. This shows a large degree of optimization, well beyond the diffraction limit, for this primary wavelength window. (c) Performance is also diffraction-limited across a broader wavelength range from 800 - 1300 nm. The longest wavelengths, 1200 nm and 1300 nm will likely be somewhat vignetted (have higher aberrations) only at the very edge of the FOV. The RMS wavefront error remains below the diffraction limit for most of the $0 - 3^\circ$ scan angle range, when the focal plane is allowed to naturally shift with wavelength. (d) The correction collar provides an additional degree of optimization. Using both parameters (adjusting the correction collar and allowing for refocusing with wavelength), the RMS wavefront error remains below the diffraction-limit for an even larger extent of the scan range for the 1200 nm and 1300 nm light. Note that this optimization increases RMS wavefront error at small scan angles, as a trade-off for a reduction at higher angles. Still, the performance remains below the diffraction limit (0.072λ). Overall, a diffraction-limited performance is attained across a broad range of wavelengths, and across a wide FOV.

135 diameters being at the back of the objective, near the threads. Realizing this problem in an early
 136 version, we redesigned the optomechanics for assembly in the middle, at the adjustable air gap
 137 surface (Surface 6). Lenses are loaded from this plane, into both halves, and then the two halves
 138 are joined. This reduced the diameter of the shoulder near the threads, and moved the largest
 139 diameter to the middle of the lens, where it can be more easily accommodated on commercial
 140 multiphoton microscopes. The resulting silhouette of the objective resembles a couasa squash,
 141 and inspired the name of the objective (Fig. 3a). The total traveling range of the adjustable air
 142 gap is 1.0 mm, corresponding to 1.3 revolutions of the correction collar, with a precision of
 143 $2.08 \mu\text{m}$ per degree. The correction collar is marked to indicate both 360-degrees around the
 144 objective, and various cover glass thicknesses. The tip of the objective is beveled at 45° to gain
 145 some clearance near the sample space.

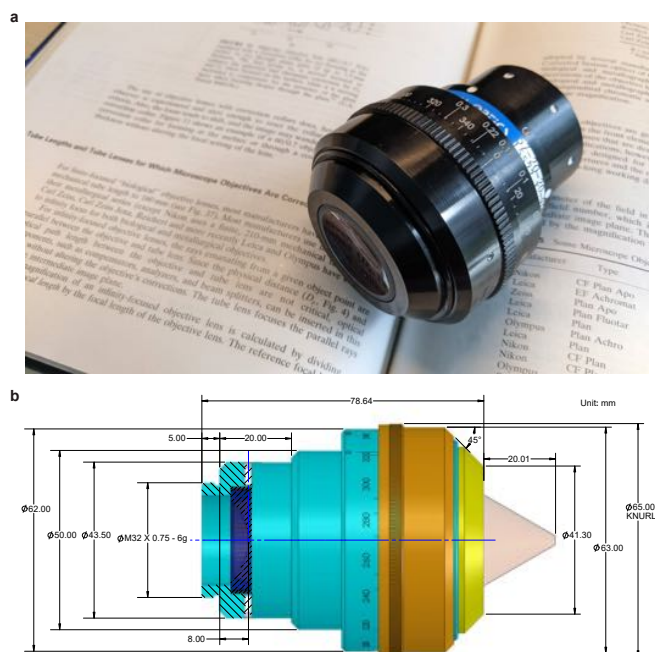


Fig. 3. The housing of the objective. (a) A photograph of the manufactured objective, and (b) the mechanical model of the objective. All dimensions are in mm unless otherwise noted.

146 3. Characterization and performance

147 3.1. Resolution, field-of-view, and light transmission

148 We characterized the real world performance of the objective using a custom two-photon scan
149 engine with a 32 mm diameter beam scanned over a $\pm 5^\circ$ range [9]. These scan parameters exceed
150 the requirements of the objective (20 mm and $\pm 3^\circ$, respectively), thus the performance should
151 be objective-limited, rather than scan engine-limited. We first measured the resolution attained
152 by the objective by taking z-stacks of 0.2 μm fluorescent beads at various positions across the
153 FOV (**Fig. 4a and 4b**). The lateral FWHM is 0.69 μm throughout the FOV, which is consistent
154 with the theoretical diffraction-limited resolution [15]. The axial resolution is 5.84 μm , again
155 providing a good match to the theoretical value, up to $\pm 2^\circ$ scan angles, and deviates by about
156 10% at 3° of scan angle. The match between the experimental measurement and the theoretical
157 calculation confirms that the NA of the objective is 0.50, as designed. This result also implies
158 that the RMS wavefront error is low.

159 We next measured the imaging FOV with a structured fluorescent sample with periodic lines
160 (5 per mm; item 57-905, Edmund Optics). When the scan angle is $\pm 3^\circ$, the images contain 10
161 lines along both the x and y directions without vignetting, indicating a 2 mm length on each axis
162 of the FOV (**Fig. 4c**). The result demonstrates that the objective has a FOV of $2 \times 2 \text{ mm}^2$ area,
163 consistent with the nominal model performance (**Fig. 2**). The FOV can be extended to $\sim 3 \times 3 \text{ mm}^2$
164 with a scan of $\pm 5^\circ$, and vignetting occurs at the corners of the field (**Fig. 4c**).

165 The broadband antireflective coating applied to the lenses was measured to transmit on average
166 99.5% of visible and near-infrared light (450 - 1100 nm) per surface. To measure the total
167 transmission of 910 nm and 532 nm light through this objective, we supplied an under-filling
168 laser beam into the objective, and measured its power before and after the objective. We found
169 that 86% of 910 nm and 91% of 532 nm light were transmitted through the objective, showing

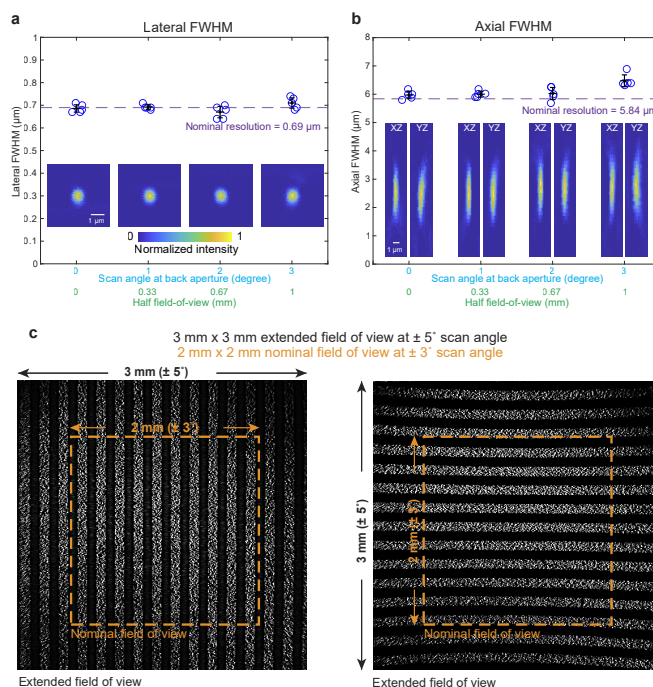


Fig. 4. Characterization of the resolution and FOV. Two-photon excitation PSF measurements were made with 0.2- μm beads embedded in agar at a depth of 350 μm covered by 170- μm thick coverslip. Z-stack images are acquired for beads at four lateral locations including on-axis, 1°, 2°, and 3° off-axis. Full-width at half-maximum (FWHM) of the Gaussian fits for measurements from the fluorescence beads laterally (a) and axially (b) are calculated and plotted. Five beads ($n=5$) at each locations are measured. Data are presented as mean values \pm S.D. Dashed lines in (a) and (b) indicates the theoretical lateral and axial FWHM, respectively. Insets in (a) and (b) show the example images of the XY, XZ, and YZ cross-sections of the measured beads at each scan angle. (c) XY images of a fluorescent calibration sample with a periodic line pattern (5 lines per millimeter) in two orientations acquired under a $\pm 5^\circ$ scan angle. Each image shows 15 lines on the top edge (left image) and on the left edge (right image), respectively, corresponding to a 3 x 3 mm FOV. Both the dashed squares show a nominal 2 x 2 mm FOV of the objective under the $\pm 3^\circ$ scan angle.

170 very high transmission throughput at both the excitation and emission wavelengths for green
 171 fluorescence protein imaging.

172 3.2. *In vivo* two-photon calcium imaging in mouse

173 After benchmarking the optical performance of the objective, we tested the performance in the
 174 target application: two-photon imaging *in vivo*. The Cousa objective was used on a range of
 175 multiphoton imaging systems, including custom-built systems and commercial systems from
 176 Bruker, Thorlabs, Neurolabware, and Sutter. Three different animal species were used as well:
 177 mouse, ferret, and tree shrew.

178 As a first test, a cranial window was implanted in a transgenic mouse with neurons expressing
 179 the genetically encoded calcium indicator GCaMP6s [16]. The Cousa objective was mounted
 180 on a custom microscope that provided a $\pm 2.6^\circ$ scan angle range and a 20 mm diameter beam
 181 at the back aperture of the objective. This system also had a 12 kHz resonant scanner for fast

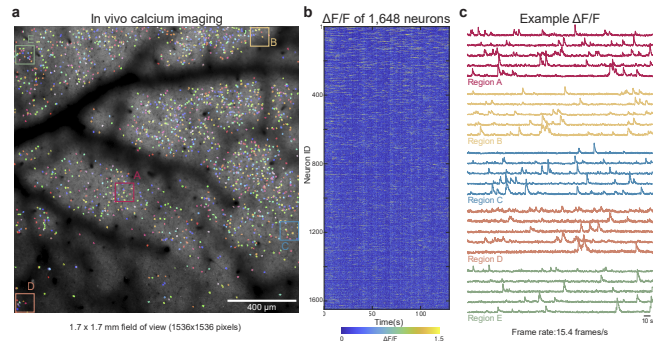


Fig. 5. *In vivo* calcium imaging. (a) Calcium indicator dynamics were imaged *in vivo* over a 1.7 x 1.7 mm FOV through a cranial window on a transgenic mouse expressing the genetically encoded fluorescent calcium indicator GCaMP6s in excitatory neurons. The color-coded regions are neurons with calcium activity identified. (b) Calcium transients ($\Delta F/F$) from 1648 neurons were detected and plotted over time. (c) Examples of $\Delta F/F$ from different regions in (a) are plotted.

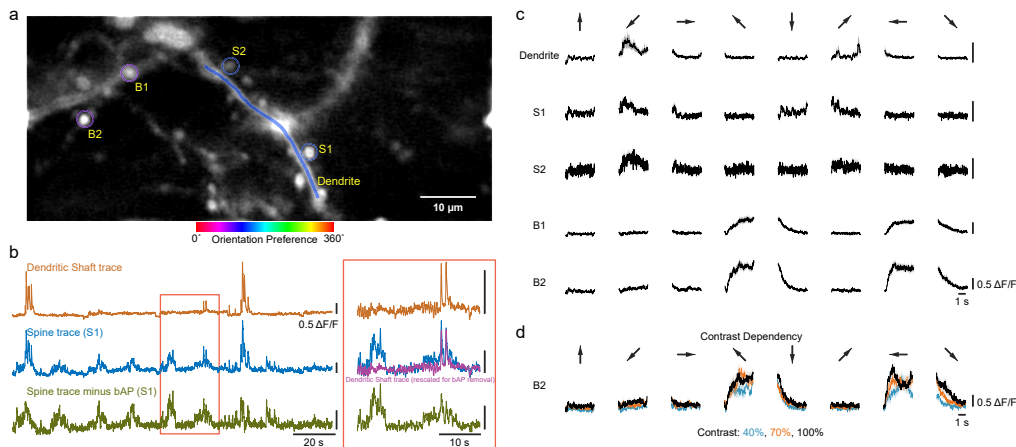


Fig. 6. *In vivo* imaging of neural processes and orientation tuning in mice. (a) *In vivo* calcium transients from neural processes were recorded through a cranial window in a mouse with ultra-sparse expression of GCaMP8m in V1. Black and white drifting gratings were presented on a display monocularly. Putative axonal boutons (B), dendritic spines (S), and dendritic shafts were clearly resolved. Color-codes show the orientation preference of each region of interest. (b) Calcium transients from the dendrite (brown) and the dendritic spine S1 before back-propagating action potential (bAP) signal is removed (blue). After the bAP signal was removed, the trace of spine S1 (green) shows many activity events that are independent from its local dendritic shaft. (*inset*) An expanded view shows further details the subtraction procedure, including a rescaled trace from the dendritic shaft (purple) for removal of the bAP signals. (c) Orientation tuned responses for were reliable for spines S1 and S2, boutons B1 and B2, and the nearby dendrite ($n = 15$ repeats per stimulus; mean in black \pm SEM in gray). (d) Responses in axonal bouton B2 varied with contrast (contrast levels of 40% in blue, 70% in orange, and 100% in black). Traces show the mean \pm SEM in shading; $n = 5$ repeats for each contrast and orientation).

182 raster scanning. First, a z-stack image series was acquired covering the volume of $1 \times 1 \times 0.5$
183 mm^3 ($X*Y*Z$) (**Video 1**). This data demonstrated that individual neurons were resolved up to
184 the depth of 0.5 mm. Next, a FOV of $1.7 \times 1.7 \text{ mm}^2$ was recorded using the full $\pm 2.6^\circ$ scan
185 angle at back aperture (**Fig. 5a, Video 2**) with 1536 scan lines, 1536 pixels per scan line, and a
186 frame rate of 15.4 frames/s. Spontaneous calcium transients were imaged from 1648 neurons
187 detected throughout the FOV (**Fig. 5b**). Calcium indicator traces from neurons across the FOV
188 exhibited high $\Delta F/F$ signals (**Fig. 5c**). These *in vivo* results demonstrate performance in the
189 target application, with a relatively large FOV, even when using relatively short pixel dwell times
190 ($\sim 28 \text{ ns}$ / pixel or ~ 2 pulses / pixel).

191 We next performed two-photon imaging of dendrites and axons in a mouse that sparsely
192 expressed GCaMP8m. Neuronal activity in the primary visual cortex (**V1**) was imaged while the
193 animal viewed black and white drifting gratings of eight different orientations ($0\text{-}315^\circ$ and 45°
194 steps). The spines and their local dendritic shaft are clearly resolved, and some putative boutons
195 are identified with distinctive calcium activity (**Fig. 6a**). Dendritic spine transients showed clear
196 independent calcium dynamics in addition to those associated with back-propagating action
197 potentials (**bAP**), demonstrating that the fluorescence signals from the spine and its parent
198 dendrites can be unambiguously extracted such that the bAP signals can be removed from the
199 spine with high fidelity (**Fig. 6b**) [6]. These identified spines, boutons, and dendrite show
200 reliable response to visual stimuli and different orientation tuning (**Fig. 6c**). Moreover, response
201 magnitude of the axonal bouton transients showed contrast-dependence (40%, 70%, and 100%),
202 further highlighting the sensitivity of the objective and performance in challenging experiments
203 (**Fig. 6d**). Taken together, these results demonstrate that the Couse objective has not only high
204 resolution for resolving minute structures in neural processes, but also sufficient two-photon
205 excitation and collection efficiency to detect fine changes in calcium transients.

206 3.3. *In vivo two-photon calcium imaging in ferret and tree shrew*

207 As previously discussed, two-photon microscopy of *in vivo* neuronal activity in larger animals can
208 be challenging due to the mechanical limitation of multiphoton objectives. Therefore, we tested
209 the Couse objective in ferrets and tree shrews. The objective was mounted on a commercially
210 available microscope (B-Scope; Thorlabs) to image calcium dynamics in neurons in ferret and
211 tree shrew V1. Injected viral particles transduced neurons to express GCaMP6s [16]. For ferret
212 imaging, wide-field imaging was used to image the vasculature and the orientation preference
213 map in V1 (**Fig. 7a**). Then, two-photon imaging through the Couse objective was used to resolve
214 neuronal activity of individual neurons within a $2 \times 2 \text{ mm}$ FOV (**Fig. 7b**). Individual neurons
215 exhibited reliable responses to visual stimuli with edges of particular orientations (**Fig. 7c**).
216 Observed two-photon orientation preferences were also consistent with their location within the
217 orientation preference map (as measured with widefield imaging).

218 In tree shrews, V1 neurons were transduced to express GCaMP6s [16]. The Couse objective
219 was used to image calcium transients (**Fig. 8a**). Individual neurons could be registered to their
220 location in the local orientation preference map (**Fig. 8b**), and reliable responses to visual stimuli
221 were resolved (**Fig. 8c**). In both ferret and tree shrew V1 imaging, the Couse objective offered
222 a larger FOV than conventional objectives, and the air immersion increased the reliability of
223 long-term imaging sessions, over which maintenance of a water interface can be unreliable,
224 especially when imaging at an angle. Together, these experiments demonstrate that the Couse
225 objective supports multiphoton imaging in ferrets and tree shrews.

226 3.4. *Simultaneous 2-photon and mesoscopic widefield imaging in awake, head-fixed* 227 *mice*

228 Many objectives used in two-photon imaging are designed for water immersion, which can
229 complicate experiments where the objective needs to be held at an angle far from vertical. In

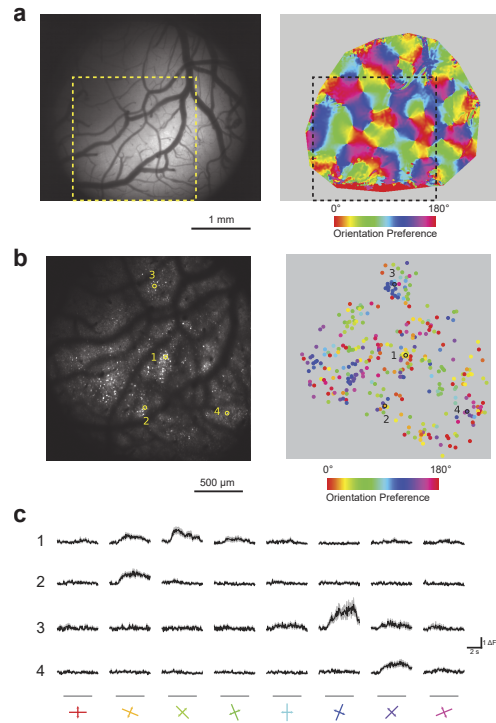


Fig. 7. Orientation preference maps in ferret. (a) Widefield epifluorescence calcium imaging of ferret visual cortex using a 4x objective. FOV (left) and orientation preference map (right). Dashed boxes denote the approximate two-photon FOV shown in (b). (b) Two-photon calcium imaging of the corresponding FOV shown in (a) using the Coussa objective. FOV (left) and scatter of cellular orientation preferences (right). (c) Orientation tuned cellular responses for presentation of oriented stimuli for the four cells shown in (b). Traces show mean (black) and SEM (gray). Horizontal gray bars denote the stimulus presentation period.

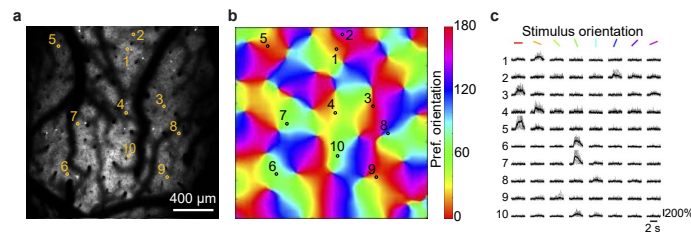


Fig. 8. Orientation preference maps in tree shrew. (a) GCaMP6s expression in a 2x2 mm FOV, 136 μm below the pia in tree shrew V1. Ten example neurons were selected. (b) Pixel-wise orientation preference map generated by two-photon imaging. (c) Orientation tuned cellular responses of the example neurons shown in (a) and (b). Traces show mean (black) and single trials (gray).

230 addition, short working distances preclude the implementation of intermediate optics between
 231 the objective and the sample. Long working distance air objectives can enable experiments such
 232 as simultaneous mesoscopic and two-photon imaging of neuronal activity [17]. In this method,

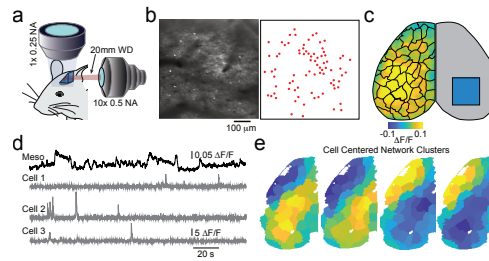


Fig. 9. Dual 2-photon/mesoscopic imaging in awake, head-fixed mice. (a) Schematic illustrating the imaging configuration. Awake, head-fixed mice ($n=2$) were placed under the objective of a widefield microscope. Cellular data were acquired through the Couda objective directing the light path through a prism placed over visual cortex. (b) Time-averaged 2-photon FOV (left) and location of imaged neurons (right) for a representative experiment. (c) Example mesoscopic imaging frame with overlaid functional parcellation boundaries. Prism location in opposite hemisphere is indicated. (d) Example time series for three representative neurons and a parcel over the visual cortex. (e) Average k means-clustered ($k=4$) cell-centered networks for 74 neurons in one animal.

233 dual asymmetric imaging pathways are used to record the activity of individual neurons relative
 234 to ongoing, large-scale dynamics across the dorsal neocortex. The Couda objective was mounted
 235 horizontally and used in conjunction with a micro prism implanted on the cortical surface (**Fig.**
 236 **9**) [17]. Compared to prior instrumentation, the Couda objective offered a larger FOV (more
 237 neurons imaged) and a higher NA (i.e., improved resolution).

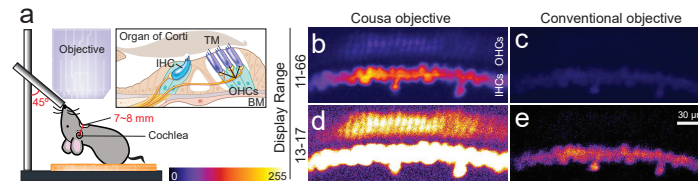


Fig. 10. *In vivo* cochlear hair cell imaging with the Couda objective and a conventional objective. (a) Illustration of the mouse position for two-photon imaging of the organ of Corti in the cochlea. IHC, inner hair cell; OHC, outer hair cell; TM, tectorial membrane; BM, basilar membrane. The color bar indicates a look-up table with a range of 0-255 for panels b-e. (b-e) Z-projection images of IHCs and OHCs with the Couda objective or a conventional objective. The display range of 11-66 or 13-17 was applied for comparison of the objectives.

238 3.5. Imaging the mouse cochlea *in vivo*

239 Challenging surgical preparations are another example of experiments requiring challenging
 240 imaging angles, ample working distances, air immersion, and optimized two-photon performance.
 241 Using *in vivo* multicellular imaging, we can directly measure neuronal responses in peripheral
 242 sensory encoding or sight [18], smell [19], taste [20] and touch [21]. However, obtaining similar
 243 *in vivo* information in the auditory system is particularly challenging. The peripheral end organ,
 244 the cochlea, is difficult to reach surgically and optically. The cochlea is located deep in the
 245 temporal bone, next to the bulla, tympanic membrane, and other structures that limit optical
 246 access. Moreover, it is a mechanosensitive, fluid-filled structure, which further complicates

247 surgical preparations and functional imaging. Recently, a novel surgical approach overcame these
248 challenges, enabling *in vivo* visualization of multiple cochlear cells while preserving hearing
249 function [21]. In this approach, a long working distance objective (> 8 mm) is required to keep the
250 neighboring hearing structures intact (**Fig. 10a**). Furthermore, an air objective is needed since
251 an air-filled middle ear cavity is critical for sound transference through the middle ear *in vivo*.
252 Water or oil coupling for the objective interrupts effective sound transfer. However, existing long
253 working distance air objectives with NA (> 0.40) have poor performance (transmission and/or
254 aberration correction) for the near-infrared wavelengths used in two-photon microscopy. Thus,
255 high laser power is required. Here, we directly compare the performance of the Cousa objective
256 with a conventional long working distance objective in this application. Two-photon images of
257 the organ of Corti were collected *in vivo* with the same laser power (30 mW) in a genetically
258 modified mouse (a hair cell-targeted Myosin15Cre line crossed with a TdTomato reporter line).
259 The image intensity of inner and outer hair cells (**IHCs and OHCs**) from the Cousa objective is
260 higher than that from a conventional objective (**Fig. 10b-e**). Specifically, the display range of
261 11-66 (arbitrary units) visualized both IHCs and OHCs with the Cousa objective, while
262 with the conventional objective cells were rarely detected (**Fig. 10b,c**). Similarly, the proper
263 display range of 13-17 for the conventional objective made the signal from the Cousa objective
264 saturated (**Fig. 10d,e**). Lastly, doubled laser power (60 mW) for improving image quality with
265 the conventional objective induced cell damage. Taken together, the Cousa objective shows a
266 much greater efficiency for cochlear *in vivo* imaging compared to a conventional objective, thus
267 enabling measurements with less laser power.

268 **4. Discussion**

269 In summary, we developed a new objective optimized to enable new multiphoton experiments.
270 The key attributes of the Cousa objective include a 20 mm working distance, air immersion, an NA
271 of 0.50, and a FOV of 4 mm² (up to 9 mm² at $\pm 5^\circ$ scanning). It is optimized for low aberration
272 imaging across the near infrared (IR) spectrum (800 - 1300 nm) for multiphoton excitation.
273 The Cousa objective is designed to readily integrate with and maximally exploit commercial
274 microscope systems, with its 20 mm diameter back aperture and M32 x 0.75 mounting threads.
275 The lens description is fully open-source, so that the community can readily duplicate, modify,
276 or simulate for their applications. The manufactured objective has been distributed to an array
277 of labs, and their results demonstrate functional and structural two-photon imaging *in vivo*. In
278 conclusion, this ready-to-use open source objective can facilitate a variety of two-photon imaging
279 experiments in a wide range of animals.

280 **5. Methods**

281 **5.1. Objective design and assembly**

282 The objective was modeled and optimized using an optical design software of OpticStudio
283 (Zemax, LLC). Tolerance analysis indicated that 90% of the completed objectives would have an
284 RMS wavefront error of 0.048λ (still well below the diffraction limit criterion of 0.072λ) with
285 commonly attained manufacturing and assembly tolerances. All lenses in the objective were
286 manufactured, aligned, and assembled in the factory of Special Optics (Denville, NJ, USA). The
287 manufacturing tolerances used were 0.005 mm total indicator runout (TIR) for decentration and
288 tilt, 0.05 mm for thickness, 4 rings for radius (power), .25 waves at 633 nm for irregularity, 0.005
289 mm for wedge, 60-40 scratch-dig, and 0.01 arc min for lens decentration.

290 **5.2. In vivo two photon imaging system**

291 All imaging was performed using two custom two-photon systems. One system equipped a 8
292 kHz resonant scanner (CRS 8 kHz, Cambridge technology) supplies a 32-mm diameter beam

293 size and $\pm 5^\circ$ scan angles at the objective back aperture. The detailed information of this system
294 can be found in this reference [9]. The other system equipped a 12 kHz resonant scanner (CRS
295 12 kHz, Cambridge technology) supplies a higher imaging frame rate with a 20-mm beam size
296 and $\pm 2.6^\circ$ scan angles at the objective back aperture. Our laser source is a Ti:sapphire pulsed
297 laser with a central wavelength at 910 nm and a 80 MHz repetition rate (Mai-Tai, Newport). The
298 image acquisition were controlled by ScanImage from Vidrio Technologies Inc. The imaging was
299 performed with a power ≤ 80 mW out of the front of the objective. No damage was observed from
300 the surface of the dura to the 500 μm depth. Assessment of damage due to laser intensity was
301 based on visual morphological changes to the appearance of the dura mater and/or continuously
302 bright cell bodies.

303 *5.3. Excitation point spread function measurements and simulations*

304 The measurement and analysis procedure were described in our previous publication in details [10].
305 To evaluate the excitation point spread function (PSF), sub-micrometer beads were imaged.
306 Sub-micrometer fluorescent beads (0.2 μm , Invitrogen F-8811) were embedded in a thick (1.2
307 mm) 0.75% agarose gel. 30 μm z-stacks were acquired, each centered at a depth 250 μm . The
308 stage was moved axially in 0.5 μm increments (Δstage). At each focal plane 30 frames were
309 acquired and averaged to yield a high signal-to-noise image. Due to the difference between the
310 refractive index of the objective immersion medium (air) and the specimen medium (water), the
311 actual focal position within the specimen was moved an amount $\Delta\text{focus} = 1.38 \times \Delta\text{stage}$ [22].
312 The factor 1.38 was determined in Zemax and slightly differs from the paraxial approximation
313 of 1.33. These z-stack images were imported into MATLAB for analysis. For the axial PSF,
314 XZ and YZ images were created at the center of a bead, and a line plot was made at an angle
315 maximizing the axial intensity spread, thereby preventing underestimation of the PSF due to
316 tilted focal shifts. For the radial PSF, an XY image was found at the maximum intensity position
317 axially. A line scan in X and Y was made. Gaussian curves were fit to the individual line scans
318 to extract FWHM measurements. The radial PSF values are an average of the X PSF and Y PSF,
319 and the axial PSF is an average of the axial PSF found from the XZ and YZ images. Excitation
320 PSF measurements were performed at locations of on axis, 1° , 2° , and 3° off axis across the FOV.
321 Data reported (Fig. 4a and 4b) are the mean \pm S.D. of 5 beads ($n=5$) at each location.

322 *5.4. Animal experiments*

323 *5.4.1. Mouse experiments in Figures 5 and 6*

324 All procedures involving living animals were carried out in accordance with the guidelines
325 and regulations of the US Department of Health and Human Services and approved by the
326 Institutional Animal Care and Use Committee at University of California, Santa Barbara. Mice
327 were housed in 12 h dark/light reverse cycle room. The temperature set-point is 74–76 $^\circ\text{F}$; the
328 low-temperature alarm is 70 $^\circ\text{F}$; the high-temperature alarm is 78 $^\circ\text{F}$. The relative humidity is
329 45% (range 30–70%).

330 For population calcium imaging, GCaMP6s transgenic mice were used, which were generated
331 by triple crossing of TITL-GCaMP6s mice, Emx1-Cre mice (Jackson Labs stock #005628)
332 and ROSA:LNL:tTA mice (Jackson Labs stock #011008). TITL-GCaMP6s mice were kindly
333 provided by Allen institute. Transgenic mice were deeply anesthetized using isoflurane (1.5–2%)
334 augmented with acepromazine (2 mg/kg body weight) during craniotomy surgery. Carprofen (5
335 mg/kg body weight) was administered prior to surgery, as well as after surgery for 3 consecutive
336 days. Glass windows were implanted over visual cortex as previously described [9]. Ca^{2+}
337 signals were analyzed using custom software [23] in MATLAB (Mathworks). Neurons were
338 segmented and fluorescence time courses were extracted from imaging stacks using Suite2p
339 (<https://suite2p.readthedocs.io/en/latest/>) [24]. Signals from neurons are a sum of neuronal and
340 neuropil components. The neuropil component was subtracted from the neuronal signals by

341 separately detecting it and subtracting it. The neuropil component was isolated using the signal
342 from an annulus region around each neuron, and then subtracted from the neuronal signal to
343 provide a higher fidelity report of neuronal fluorescence dynamics. An exponential moving
344 average with a moving window size of 5 samples (0.32 s) was used to reduce the baseline noise
345 in the traces displayed (**Fig. 5c**).

346 For dendrite calcium imaging, adult (> 8 weeks) C57Bl/6 mice of both sexes (Jackson Labs)
347 were used. A 4-mm diameter craniotomy was performed over visual cortex as previously
348 described [25]. Briefly, mice were premedicated with a sedative, acepromazine (2 mg/kg body
349 weight, i.p.), after which they were deeply anesthetized using isoflurane (2-3% for induction,
350 1-1.5% for surgery). The mouse's body temperature was monitored and actively maintained using
351 an electronic heat pad regulated via rectal probe. Carprofen (5 mg/kg body weight, s.c.) was
352 administered preoperatively, and lidocaine solution containing epinephrine (5 mg/kg body weight
353 s.c.) was injected locally before and after the scalp excision. The scalp overlaying the right
354 visual cortex was removed and a custom head-fixing imaging chamber with a 5-mm diameter
355 opening was mounted to the skull with cyanoacrylate-based glue (Oasis Medical) and dental
356 acrylic (Lang Dental). Mice were mounted on a custom holder via the headplate chamber, which
357 was filled with a physiological saline containing (in mM) 150 NaCl, 2.5 KCl, 10 HEPES, 2
358 CaCl₂ and 1 MgCl₂. A craniotomy was performed using carbide and diamond dental burs on
359 a contra-angle handpiece (NSK). adeno-associated viral (AAV) vectors were injected in to V1
360 under continued isoflurane anesthesia as previously described [25–27]. Briefly, 1:1 mixture of
361 pENN.AAV.CamKII 0.4.Cre.SV40(AAV1; Addgene #105558; diluted at 1:20,000 in phosphate
362 buffered saline (PBS)) and pGP.AAV.syn.GLEX.jGCaMP8m.WPRE (AAV1; Addgene #162378;
363 original concentration at ~ 10¹³ vg/mL) viral particles were injected (80 nL per site; 1 site per
364 animal) into V1 with a pulled-glass capillary micropipette using a Nanoliter 2010 controlled by a
365 microprocessor, Micro4 (World Precision Instruments), at 15 nL per min. The glass pipette was
366 left in place for 5 mins before retracting to avoid the backflushing of the injected solution. The
367 cranial window was then sealed with a glass cranial plug made up of 4-mm and 3-mm circular
368 coverslips (Warner Instruments) stacked in tandem with a UV-curing optical adhesive (NOA61,
369 Norland). Two-photon imaging of Ca²⁺ transients indicated by GCaMP8m was performed
370 starting 4-6 weeks after AAV injection, using a custom-built two-photon microscope used in prior
371 studies [25, 28]. Frame scans were acquired using ScanImage [29] at 58.2 frames per second,
372 512x256 pixels; 31000 frames total per visual stimulation session.

373 Visual stimuli were presented on a 7" monitor (60 Hz refresh rate) placed 12 cm away from
374 the animal's eye. To assess orientation tuning of the dendritic shaft, spines, and putative axonal
375 boutons, full field square gratings at 40%, 70%, and 100% contrasts (0.04 cycles per degree at 2
376 Hz) were presented in 8 directions (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°) for 5 trials. Each
377 grating drifted for 4 seconds. A notch filter centered at 2 Hz (± 0.5 Hz bandwidth) was used to
378 remove a small amount of light leakage from the stimulus monitor into the imaging pathway.

379 To functionally map visual cortex for targeted injection of viral vectors, ISOI was performed
380 using a custom macroscope and a CCD camera as previously described [25, 30]. Retinotopic
381 maps were used to locate V1. The pial vasculature map relative to the retinotopic maps was used
382 to guide targeted injections into V1.

383 5.4.2. Ferret and tree shrew experiments in Figures 7 and 8

384 All experimental procedures were approved by the Max Planck Florida Institute for Neuroscience
385 Institutional Animal Care and Use committee and were performed in accordance with guidelines
386 from the U.S. National Institute of Health. We used one juvenile female ferret from Marshal
387 Farms and one adult male tree shrew for this study.

388 Viral transduction and terminal imaging in L2/3 of the anesthetized ferret and tree shrew
389 were performed as previously described [31, 32]. Briefly, we expressed GCaMP6s by direct

390 microinjection of AAV2/1-hSyn-GCaMP6s-WPRE-SV40 (Addgene, 100843-AAV1, Titer: 2.5^{13}
391 GC/mL) into the visual cortex. Subsequently a cranial window was implanted over visual cortex
392 and imaged. An injection into the visual cortex of the ferret was made at P21, and imaging was
393 performed at P42. Imaging in the tree shrew occurred 16 days after viral transduction.

394 Two-photon imaging of GCaMP6s was performed with a Bergamo II series microscope
395 (Thorlabs) equipped with an 8 kHz resonant-galvo scanner and driven by a Mai-Tai DeepSee
396 laser or Insight DS+ (Spectra-Physics) at 910 nm or 920 nm respectively. Average excitation
397 power at the exit of the objective ranged from 40 to 60 mW. The microscope was controlled by
398 ScanImage (MBF Bioscience). Images were acquired at 15 Hz (1024x1024 pixels in the ferret,
399 512x512 pixels in the tree shrew). Widefield epifluorescence imaging of GCaMP6s in the ferret
400 was achieved with a Zyla 5.5 sCMOS camera (Andor) controlled by μ Manager [33] through a 4x
401 air-immersion objective (Olympus, UPlanFL 4x N/0.13NA) and images were acquired at 15 Hz
402 with 4x4 binning to yield 640x540 pixel images.

403 Visual stimuli were presented on an LCD screen using PsychoPy (v1.85) [34]. The monitor (30
404 cm x 52 cm, 1920 x 1080 pixels, 120 Hz refresh rate) was placed 25 centimeters in front of the
405 animal. To evoke orientation-specific responses, full field square gratings at 100% contrast were
406 presented in 16 directions (8 orientations) for 10 trials (ferret) or 8 trials (tree shrew). Square
407 gratings were presented to the ferret at 0.06 cycles per degree and 4 Hz and in the tree shrew at
408 0.4 cycles per degree and 2 Hz. In addition, “blank” stimuli of 0% contrast were also presented.
409 All stimuli were randomly interleaved and presented for 4s followed by 6s of gray screen (ferret)
410 or 2s followed by 3s of gray screen (tree shrew). Timing for visual stimuli and imaging were
411 recorded using Spike2 (v7.11b, CED; Cambridge UK).

412 Data analysis in the ferret was performed as previously described using custom written scripts
413 in Python and ImageJ [35]. For both widefield and epifluorescence imaging, we corrected brain
414 movement during imaging by maximizing phase correlation to a common reference frame. In
415 widefield epifluorescence imaging, the ROI was drawn manually around regions where robust
416 visually evoked activity was observed. For analysis, all images were spatially downsampled
417 by a factor of 2 to yield 320x270 pixels. Slow drifts in fluorescence intensity were eliminated
418 by calculating the $\Delta F/F = (F - F_0)/F_0$. Baseline fluorescence (F_0) was calculated by applying a
419 rank-order filter to the raw fluorescence trace (10th percentile) with a rolling time window of 60s.
420 Responses were filtered with a spatial band-pass filter with low-pass cutoff defined as 50 μ m and
421 high-pass filter cutoff as 3200 μ m. Preferred orientation was computed by taking the vector sum
422 of the median-trial response over the stimulus period for each orientation.

423 For analysis, ROI were chosen semi-automatically (Cell Magic Wand v1.0) and fluorescence
424 was computed by averaging all pixels within the ROI [26]. The $\Delta F/F$ for each ROI was computed,
425 and F_0 was calculated by applying a rank-order filter to the raw fluorescence (20th percentile)
426 over a rolling time window (60 s). Stimulus-evoked responses were calculated as the average
427 $\Delta F/F$ over the entire stimulus period, and orientation preferences were computed by fitting a von
428 Mises distribution to the trial-median response for each stimulus orientation.

429 Data analysis and motion correction in the tree shrew was performed using custom code
430 written in Matlab (Mathworks) or Java package for running ImageJ within Matlab (Miji). For
431 network-level analysis, the fluorescence signal for each pixel was calculated as $\Delta F/F$, where F_0
432 is the baseline fluorescence signal averaged over a 1 s period immediately before the start of visual
433 stimulus, and F is the fluorescence signal averaged over the period of the stimulus. Responses to
434 the stimulus set were fitted with a Gaussian to determine the preferred orientation and generate a
435 pixel-based orientation preference map. For analysis at the neuronal level, regions of interest
436 (ROIs) corresponding to visually identified neurons were drawn manually using ImageJ. The
437 fluorescence of each ROI was measured by averaging all pixels within the ROI.

438 5.4.3. Dual imaging experiments in Figure 9

439 All animal handling and experiments were performed according to the ethical guidelines of the
440 Institutional Animal Care and Use Committee of the Yale University School of Medicine. Brain-
441 wide expression of GCaMP6s was achieved via neonatal sinus injection of AAV9-Syn-GCaMP6s
442 into c57/Bl6 mice, as described previously [17, 36]. After reaching adulthood (P60), the skin and
443 fascia over the skull were removed under isoflurane anesthesia and the animal was implanted
444 with a custom titanium headpost and a microprism (5mm per side, Tower Optics) placed over the
445 right visual cortex in a small craniotomy, bonded with a thin layer of dental cement (Metabond,
446 Parkell).

447 Imaging experiments were carried out in awake mice head-fixed over a freely-moving wheel
448 placed under the microscope objective. Widefield calcium imaging was performed using a Zeiss
449 Axiozoom with a PlanNeoFluar objective (1x, 0.25 NA). Epifluorescent excitation was provided
450 by an LED bank (Spectra X Light Engine, Lumencor) strobing 395 nm and 470 nm light, for
451 hemodynamic correction and calcium imaging, respectively [36]. Emitted light was collected
452 via sCMOS camera (Orca-Flash V3, Hamamatsu), with images acquired at 512 x 512 pixel
453 resolution and 10 frames per second. Data were pre-processed for hemodynamic correction and
454 normalized to $\Delta F/F$ values as previously described [2]. Functional parcellation of cortical areas
455 was carried out using local selective spectral clustering (LSSC, [36, 37]) to obtain a time series
456 of fluorescence signal for each parcel.

457 Two-photon imaging was performed using a resonant-galvo scanning microscope (MOM,
458 Sutter Instruments) coupled to our custom air-coupled, long-working distance objective (10x, 0.5
459 NA). Excitation was provided by a titanium-sapphire laser (MaiTai, SpectraPhysics) tuned to 920
460 nm. Light was directed into the brain after being reflected 90° by the implanted prism. Emitted
461 light was collected by a gallium arsenide-phosphide detector (Hamamatsu) with images acquired
462 at 512 x 512 pixel resolution and 30 frames per second. Data were motion corrected using
463 NoRMCorre [38], and regions of interest (ROIs) corresponding to single cells were manually
464 selected, neuropil-corrected, and normalized to $\Delta F/F$ values using custom software written in
465 Matlab (Mathworks).

466 We calculated cell-centered networks (CCNs) to quantify the relationship between activity in
467 single neurons and the large-scale cortical network in the contralateral hemisphere as described
468 previously [17]. Briefly, we evaluated the correlation coefficients between time series related to p
469 mesoscopic parcels and n time series related to cells to obtain C , a $p \times n$ matrix. We viewed each
470 column of C as a compact representation of synchrony between the dynamics of each cell and
471 the dynamics of the widefield signal and then clustered these vectors using the k means ($k=4$)
472 function in Matlab. We obtained the centroid map of each cluster as the average correlation
473 coefficients of all cells related to a specific cluster:

474 We then superimposed each centroid onto the full cortex parcellation to yield the average
475 images in Figure 9.

476 5.4.4. Cochlea imaging in Figure 10

477 Animal studies were carried out according to the protocols approved by the Institutional Animal
478 Care and Use Committee at Stanford University (APLAC-14345). Four weeks old male mouse
479 from Ai14tdTomato (JAX: 007908) x Myosin15Cre [39] breeding was used for cochlear hair cell
480 imaging. The mouse was anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg).
481 Anesthesia level was assessed by signs of movement or withdrawal reflex before the application
482 of supplementary anesthetic. Mouse surgery and positioning for *in vivo* cochlear imaging was
483 performed by the method described previously [21]. *In vivo* cochlear imaging was performed
484 using a modified commercial two-photon microscope (Ultima, Bruker) with long working distance
485 air objectives (Cousa objective; TU Plan ELWD 20X, NA 0.4, WD 19mm, Nikon Instruments
486 Inc.). A Ti:sapphire laser was used with wavelength 920 nm and power 30 mW (Chameleon,

487 Coherent Inc.). The projected images (**Fig. 10b-e**) were acquired in an apical hair cell location
488 (8-10 kHz) by collecting z-series 40 images with 2 μm intervals.

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499 **Contributions.** KN motivated the project, and worked with CHY and SLS to develop the specifications.
500 CHY designed the objective, coordinated fabrication, and characterized it. YY and CHY performed the
501 mouse imaging in Fig. 5. ITS and LA, along with CHY and YY, performed the dendritic spine and bouton
502 imaging in Fig. 6. JTC, JB, and DF performed the ferret and tree shrew imaging in Figs. 7 and 8. AHM,
503 HD, JAC, and MH performed the dual-imaging in Fig. 9. JK and AR performed the cochlea imaging in Fig.
504 10. GBS provided feedback on mechanical design, usability, and performance. CHY and SLS wrote the
505 paper with contributions from all authors. SLS led the project.

506 **Disclosures.** The design of the objective is not patented, and it will not be patented in the future. All
507 designs originating in this report are free for reuse, no licensing or material transfer agreements are required.
508 Notification is not required either, but only humbly requested. SLS is a paid consultant for companies that
509 sell optics and multiphoton microscopes. There are no other competing interests.

510 **Data Availability Statement.** The design of the objective is fully open source and full specification are
511 detailed in this report. Data underlying the results from imaging in animals are available from the authors
512 upon request.

513 **Supplemental videos.**

514 Video 1: Z-stack of *in vivo* calcium imaging. The z-plane range spans from the brain surface to the depth
515 of 500 μm . Frame size = 1024 x 1024 pixels. Imaging power = 80 mW.

516 Video 2: *In vivo* calcium imaging over a 1.7 x 1.7 mm FOV. Frame rate = 15.4 frames/s. Frame size =
517 1536 x 1536 pixels. Imaging power = 60 mW.

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