Miniaturized head-mounted microscope for whole-cortex mesoscale imaging in freely behaving mice

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The advent of genetically encoded calcium indicators, along with surgical preparations such as thinned skulls or refractive-index-matched skulls, has enabled mesoscale cortical activity imaging in head-fixed mice. However, neural activity during unrestrained behavior substantially differs from neural activity in head-fixed animals. For whole-cortex imaging in freely behaving mice, we present the ‘mini-mScope’, a widefield, miniaturized, head-mounted fluorescence microscope that is compatible with transparent polymer skull preparations. With a field of view of 8 × 10 mm² and weighing less than 4 g, the mini-mScope can image most of the mouse dorsal cortex with resolutions ranging from 39 to 56 µm. We used the mini-mScope to record mesoscale calcium activity across the dorsal cortex during sensory-evoked stimuli, open field behaviors, social interactions and transitions from wakefulness to sleep.

Results

Mini-mScope design. Design of the mini-mScope was constrained by three criteria. First, the overall weight of the device needed to be less than approximately 15% of the mouse body weight (<4.0 g) to permit free behavior and mobility. Second, the device needed to image most of the dorsal cortex of the mouse. Third, the imaging resolution needed to be sufficient to image mesoscale activity dynamics across the whole FOV. We recently developed See-Shells-transparent polymer skulls that can be chronically implanted on mice and provide access to 45 mm² of the dorsal cortex in head-fixed animals. In this study, we adapted the See-Shell to fit the mini-mScope by incorporating a planar top surface in the three-dimensional (3D) printed frame, eliminating the head-post and incorporating three tabs. Disk magnets were embedded in the two lateral tabs to align with disk magnets on the bottom of the mini-mScope or a protective cap (Fig. 1a). A short sleeve surrounding the mini-mScope base and the posterior tab in the bottom housing constrains the mini-mScope laterally once mounted on the See-Shell. The tab at the back of the See-Shell frame was used to gently restrain the mouse during removal of the protective cap and attachment of the mini-mScope. Attaching the mini-mScope typically takes less than 5 s and can be done without anesthetizing the mouse.

The mini-mScope consists of two interlocking computer numeric control (CNC)-machined Delrin housings (Fig. 1a). Three blue light-emitting diodes (LEDs) paired with an excitation filter are installed into three illumination arms in the bottom housing (Fig. 1b). A green LED provides illumination for reflectance measurements. A biconvex lens and emission filter are embedded in the central shaft of the bottom housing. A complementary metal oxide semiconductor (CMOS) sensor is mounted on the top...
housing, which is designed to slide along the central square shaft of the bottom housing to adjust focusing. The three blue LEDs are wired in series and the wires to power the green and blue LEDs are routed through a commutator to alleviate torsional strain on the device. Mice exhibited a repertoire of behaviors, including grooming and rearing, indicating their comfort with the mini-mScope (Fig. 1b and Supplementary Video 1).

The weight of the device is 3.8 g, which is heavier than some miniaturized microscopes\textsuperscript{17}, but comparable to devices developed for volumetric imaging\textsuperscript{19}. Based on computer-aided design (CAD) simulations, we estimate that the center of gravity is approximately 24.7 mm above the mouse head. Detailed instructions for assembling the mini-mScope are provided in Supplementary Fig. 1 and Supplementary Note 1.

\textbf{Mini-mScope optical performance.} In contrast to typical imaging through a reinforced intact skull, the mini-mScope images the cortex through a transparent polymer skull. Qualitatively, imaging the cortex of a Thy1-GCaMP6f mouse\textsuperscript{23}, which expresses fluorescent reporters of calcium activity in excitatory neurons, with the mini-mScope allowed us to achieve a high optical resolution across the FOV (Fig. 1c) compared to imaging the cortex through the intact skull. The optical resolution was estimated to be \textit{d} = 2 nm, which is comparable to the diffraction limit of a clean, highly transparent skull.

\textbf{Fig. 1} | The mini-mScope: a miniaturized head-mounted microscope for whole-cortex mesoscale activity mapping in freely behaving mice. \textbf{a}, Photograph of the fully assembled mini-mScope and the corresponding See-Shell implant. Scale bar, 5 mm. \textbf{b}, Left: CAD rendering of the mini-mScope showing the internal components. The device is attached to the See-Shell implant via magnets. Three blue LEDs are attached to a filter holder that has a 480-nm excitation filter to excite GCaMP6f in the cortex. The green LED is used to obtain reflectance measurements for hemodynamic correction. The resulting emission signals (approximately 520 nm) are focused through a collector lens and passed through an emission filter onto a CMOS sensor that can be focused manually. Scale bar, 5 mm. Right: a still image of a mouse bearing the mini-mScope and engaging in natural behavior. \textbf{c}, Fluorescence image of the whole dorsal cortex of a Thy1-GCaMP6f mouse captured by the mini-mScope. Scale bar, 2 mm. The image is representative of \textit{n} = 29 mice. \textbf{d}, Comparative image of a head-fixed Thy1-GCaMP6f mouse imaged using a standard epifluorescence macroscope through an intact-skull preparation. Scale bar, 2 mm. The image is representative of \textit{n} = 2 mice. \textbf{e}, Left: resolution test target overlaid onto the mini-mScope FOV. Right: resolutions obtained within each specified grid. \textbf{f}, Image of fluorescein dye-infused agar phantom captured by the mini-mScope. The colored lines indicate mediolateral sections along which illumination profiles were obtained FOVs. \textbf{g}, Plot of normalized illumination profiles from the sections denoted in \textbf{f}.
skull using an epifluorescence macroscope (Fig. 1d). To measure the resolution of the mini-mScope, we imaged a 1951 USAF resolution test target positioned at eight different locations across the FOV (Fig. 1e). At a location corresponding to approximately −5.5 mm anteroposterior, approximately 0 mm mediolateral with respect to bregma, lines in group 3 and element 6 are clearly visible, indicating a resolution of 39.36 µm at this location. Since a single biconvex lens was used to image a convex surface, not all areas of the cortex are in focus and the optical resolution varied. The top housing was adjusted to obtain the best focus at approximately 1 mm lateral to...
Fig. 3 | Sensory stimulus-evoked responses imaged by the mini-mScope. 

**a**, Schematic of an anesthetized Thy1-GCaMP6f mouse with a vibrational stimulus applied to the right hind limb. 

**b**, Composite of a raw grayscale image of the brain and the pseudocolor frame where the largest average $\Delta F/F$ occurred within the 1-s stimulus period. The white dashed circle indicates the ROI analyzed in **d** and **e**. The red dot indicates bregma. Scale bar, 3 mm. 

**c**, Montage of average cortical calcium response to the vibration stimulus. Scale bar, 2 mm. 

**d**, $\Delta F/F$ traces of the contralateral ROI drawn in **b**. The red line denotes the average traces, the gray lines denote individual trials. The black bar shows the time and duration of the vibrational stimulus. 

**e**, $\Delta F/F$ traces of the ipsilateral ROI drawn in **b**. The blue line denotes the average response, the gray lines denote each individual trial. 

**f**, Peak $\Delta F/F$ within 1 s of vibration stimulus presentation ($n = 20$ trials in one mouse; data are representative of 3 mice). **P < 0.01, P = 9.1 \times 10^{-3},$ two-sample t-test. 

**g**, Schematic of a mouse with a visual stimulus applied to the left eye. 

**h**, Composite of a raw grayscale image of the brain and the pseudocolor frame where the largest average $\Delta F/F$ occurred within the 1-s stimulus period. The white dashed circle indicates the ROI analyzed in **j** and **k**. Data were acquired in the same mouse as the data in **a–f**. 

**i**, Montage of the average cortical calcium response to the visual stimulus. Scale bar, 2 mm. 

**j**, $\Delta F/F$ traces of the contralateral ROI drawn in **h**. The red line denotes the average trace, the gray lines denote each individual trial. The black bar shows the time and duration of the visual stimulus. 

**k**, $\Delta F/F$ traces of the ipsilateral ROI drawn in **h**. The blue line denotes the average ipsilateral response, the gray lines denote the individual trials ($n = 20$ trials in one mouse; data are representative of 3 mice). 

**l**, Peak $\Delta F/F$ within 1 s of visual stimulus presentation. The bolded line corresponds to the average peak response whereas the gray lines indicate the peak response for each trial. **P < 0.01, P = 1.31 \times 10^{-5},$ two-sample t-test.
the midline, resulting in resolution ranging from 39 µm along the midline to 55.6 µm more laterally, which was sufficient for observing mesoscale calcium activity.

The mini-mScope's array of three blue LEDs paired with excitation filters delivered an approximate cumulative 31 mW of power to the brain (Supplementary Fig. 2). Two of the blue LEDs were oriented at 30 degrees with respect to the optical axis. The third, located at the anterior of the bottom housing, was oriented at 25 degrees with respect to the optical axis. The LEDs were revolved around the optical axis at angles of 90, 225 and 315 degrees (Supplementary Fig. 2b). A green LED located at the posterior of the bottom housing at an orientation of 55 degrees with respect to the optical axis delivered approximately 0.22 mW of power and was used for reflectance measurements. We imaged fluorescein dye-infused agar gel using the mini-mScope to investigate illumination uniformity (Fig. 1f). The normalized light intensity decreased by 56%, 53.3% and 46.2% compared to the maximum intensity, at the mediolateral lines at 1.4 mm anteroposterior, −2.8 mm anteroposterior and −4.2 mm anteroposterior, respectively (Fig. 1g). The greatest reduction of illumination from the maximum did not exceed 60%. These metrics are comparable to the performance of previously developed large FOV scopes and allowed signals obtained from all pixels to be well within the dynamic range of the CMOS sensor.

The CMOS sensor captures images of the cortex alternatively illuminated by the green LED for reflectance imaging and the blue LEDs for epifluorescence imaging. A trigger circuit uses time stamps of CMOS frame acquisitions to precisely switch between the blue LEDs and green LED (Supplementary Fig. 2c–e). The circuit and LEDs have first-order dynamics with the blue LEDs and green LED having time constants (to reach 66.66% peak power) of 1.79 ± 0.31 ms and 1.97 ± 0.10 ms, respectively. The blue LEDs and green LED were switched on for 20 ms and 4 ms, respectively, starting after the CMOS sensor initiated each frame capture. The power intensity of the LEDs had a slow drift in average intensity value lasting approximately 2 min after which they equilibrated (Supplementary Fig. 2f and Supplementary Note 2). Therefore, we allocated a 2-min warmup period for the blue LEDs during each experiment for the power intensity to stabilize before data collection.

Comparison with a conventional epifluorescence macroscope.

We next compared the mini-mScope's imaging capabilities to a conventional epifluorescence macroscope. Green reflectance channel imaging allowed correction of the calcium fluorescence signals for hemodynamic effects (Fig. 2f,g). We also benchmarked the signal-to-noise ratio (SNR) of the two instruments by imaging stimulus-evoked activity. The mini-mScope has a reduced SNR due to the lower quantum efficiency of the mini-mScope’s CMOS sensor (Supplementary Fig. 3 and Supplementary Note 3). Furthermore, the mini-mScope performed imaging through a modified intact skull (Supplementary Fig. 4 and Supplementary Note 4). These results demonstrate that the mini-mScope can acquire calcium signals that are comparable to a conventional epifluorescence macroscope.

Imaging sensory stimulus-evoked responses across the cortex.

Stimulating distinct sensory pathways evokes neural activity in specific primary sensory areas located within the dorsal cortex. Providing a mouse under light (<1%) isoflurane anesthesia with a series of brief vibrational stimuli (1 s long, 100 Hz) to the right hind limb (Fig. 3a), evoked robust calcium activity in the contralateral hind limb region of the somatosensory cortex within 500 ms of the onset of the stimulus (Fig. 3b,c). The peak poststimulus response was 1.68 ± 0.49% ΔF/F (Fig. 3d, n = 17 trials in 1 mouse). In comparison, the peak poststimulus response on the ipsilateral side was significantly lower (0.70 ± 0.34% ΔF/F, Fig. 3e; α = 0.05 for significance, P = 9.1 × 10⁻⁴, paired t-test). We presented the same mouse with 100-ms-long flashes of white light to the left eye (Fig. 3g), which evoked a robust increase in calcium activity in the contralateral visual cortex (Fig. 3h,i). The peak poststimulus response was 1.7 ± 0.32% ΔF/F (Fig. 3j; n = 18 trials). In comparison, the peak poststimulus response on the ipsilateral visual cortex was significantly lower (0.38 ± 0.057% ΔF/F, Fig. 3k; α = 0.05 for significance, P = 1.31 × 10⁻⁴, paired t-test). Thus, the mini-mScope can reliably measure evoked responses to varied sensory stimuli in both hemispheres of the dorsal cortex. We performed experiments to test whether visual and auditory stimulus-evoked activity could be measured in mice freely locomoting in an open field. The responses were more variable owing to the awake state; nevertheless, we obtained distinct responses (Supplementary Fig. 5 and Supplementary Note 5).

Effect of mounting a mini-mScope on behavior and imaging stability.

We next assessed if either implanting the See-Shell or mounting the mini-mScope affected behavior in both short-term and long-term experiments. We found that mice implanted with the See-Shell and mice implanted with the See-Shell and fitted with the mini-mScope exhibited similar measures of agility and locomotion compared to control mice (Supplementary Fig. 6 and Supplementary Note 6). We also assessed the stability of the images captured using the mini-mScope during free behavior (Supplementary Video 2). The absolute maximum x and y displacements of the FOV were 21.0 ± 21.8 µm and 14.4 ± 17.1 µm, respectively (Supplementary Note 7).
Fig. 5 | Combined electrophysiological recording and mesoscale imaging of brain activity during wakefulness and sleep. **a**, Montages of change in glutamate activity over a 1-s period during awake, REM and non-REM sleep states. Scale bar, 2 mm. **b**, Representative traces of raw glutamate signals from V1, hippocampal LFP and video-based movement signals during the states of waking, non-REM and REM sleep in a freely moving mouse. F0 is the baseline glutamate signal calculated by averaging the fluorescence over the entire recording time. **c**, Magnification of the glutamate, LFP and movement signals selected in **b**, d. Grouped mean raw glutamate signal from the entire cortex (normalized to baseline) during wakefulness, non-REM and REM sleep (n = 3 mice, paired Friedman nonparametric test; post hoc multiple comparison with Dunn’s correction). P < 0.05, P = 0.02 wakefulness versus REM. **e**, Spectral analysis of glutamate signal in the retrosplenial cortex (RS) in the three states. **f**, Correlation maps of cortical activity between quiet wakefulness, non-REM and REM sleep on two different days. The cortex was divided into 21 ROIs. **g**, Mean correlation of cortical activity during waking, REM and non-REM sleep (n = 3 mice). **h**, Glutamate activity, hippocampal LFP frequency spectrogram and animal movement tracking during transition from REM to wakefulness. **i**, Montage of cortical glutamate changes during the transition shown in **h**. Scale bar, 2 mm.
Fig. 7 and Supplementary Note 7). These displacements could be corrected for digitally using the moco (MOtion CORrector) correction algorithm. We also observed large changes (up to 25%) in diameter of the superior sagittal sinus (Supplementary Fig. 8 and Supplementary Note 7). These variations could introduce artifacts in the analysis of the calcium signals. Thus, we removed areas close to the superior sagittal sinus from the FOV before the analyses.

Mapping cortical functional connectivity during open field exploration and social interactions. We used the mini-mScope to examine functional connectivity between cortical areas during open field behavior of solitary mice and during social interactions with a companion mouse (Fig. 4a,b). We segregated solitary open field trials into four types of behaviors: periods when mice were still, moving, grooming or rearing. On average, mice spent 70.6 ± 9.0% of the time remaining still (n = 11 trials, 3 mice), while they spent 20.7 ± 7.51% of the time moving within the arena. Grooming and rearing were less frequent and shorter in duration, accounting for 7.7 ± 6.02% and 1.04 ± 1.17% of the time, respectively (Fig. 4c). To study social behavior, we allowed mice bearing the mini-mScope to first explore the arena before we introduced a companion mouse of the same sex (n = 8 trials, 8 mice; Supplementary Video 3). Mice spent 41.3 ± 19.5% of the time socially interacting with each other, including touching whiskers or the body (Fig. 4d).

We constructed hemodynamics-corrected seed pixel correlation maps of the cortex from the calcium activity videos during open field behavior. We analyzed maps regarding six seeds within the motor cortex (M1), forelimb, hind limb and barrel cortex (BC) areas in the somatosensory cortex, the retrosplenial cortex (RSC) and the visual cortex (VC) (Fig. 4e,f). Correlations between the seed locations changed when the animal was moving versus still. Between the seeds, correlations increased, particularly between those located within the somatosensory cortex. The area of the left hemisphere of the cortex that is highly correlated regarding a given seed location increased for all seeds analyzed when the animal was moving (Fig. 4g); these increases were significantly higher for seeds located at the M1, hind limb, forelimb, VC and RSC (n = 11 trials, 3 mice, α = 0.05 for significance; P = 4.6 × 10⁻³ M1, P = 6.3 × 10⁻⁴ forelimb, P = 3.8 × 10⁻¹ hind limb, P = 4.6 × 10⁻³ RSC, P = 0.10 BC, P = 1.1 × 10⁻¹ VC, Mann–Whitney U-test). Overall, movement induced increased variance in inter-seed correlations (Fig. 4h).

Similarly, we constructed seed pixel correlation maps for mice engaging in social behaviors (Fig. 4i). Intracortical connectivity was increased during times when mice were engaged in social behaviors (Fig. 4i–k; n = 8 mice, α = 0.05 for significance, P = 0.048 M1, P = 0.17 forelimb, P = 0.058 hind limb, P = 0.091 RSC, P = 0.082 BC, P = 0.37 VC, Mann–Whitney U-test). These results demonstrate the utility of the mini-mScope to study functional connectivity during behaviors that are unique to freely behaving mice.

Imaging glutamate release dynamics during wakefulness and natural sleep. As a final demonstration, we used the mini-mScope to measure dynamic changes in extracellular glutamate release in the cortex during transition from wakefulness to natural sleep. Much of the previous work studying extracellular glutamate release has been done using fixed potential amperometry or optical imaging in head-fixed mice. Inducing sleep in head-fixed mice is challenging and typically requires sleep deprivation, which can alter the overall sleep structure and patterns of rapid eye movement (REM) and non-REM sleep. The flexibility of the See-Shells allowed us to incorporate local field potential (LFP) recording electrodes in the dorsal hippocampus in Ems-CaMKII-Ai85 mice expressing iGluSnFR in glutamatergic neocortical neurons. We allowed mice to naturally transition to sleep in their home cage and recorded glutamate activity across the whole dorsal cortex during wakefulness, REM sleep and non-REM sleep (Fig. 5). Hippocampal LFP indicated transition to non-REM sleep characterized by high amplitude slow waves (0.5–4 Hz) and subsequently REM sleep characterized by theta band activity (7–9 Hz; Fig. 5b,c,h). Consistent with previous studies, spontaneous cortical activity patterns during quiet wakefulness and non-REM sleep were highly synchronized across hemispheres. In addition, cortical activity changes were not necessarily due to global changes in state and were instead composed of complex local activity patterns (Fig. 5a). The transition from wakefulness to non-REM and REM sleep resulted in decreased fluorescence, indicating reduced cortical glutamate activity (Fig. 5a–d). We also observed a reduction of slow cortical glutamate fluctuations during REM sleep (Fig. 5e). Correlation analysis of cortical activity revealed that connectivity decreases in REM sleep compared to quiet wakefulness and non-REM sleep (Fig. 5f,g). Moreover, consistent with previous studies, the strength of functional connectivity was less in non-REM sleep compared to quiet wakefulness. The mini-mScope attachment also allowed us to study the transition from REM sleep to wakefulness, wherein we observed increased glutamate activity across the cortex (Fig. 5h,i).

Discussion

We have introduced a neurotechnology for mesoscale activity mapping of the dorsal cortex in freely behaving mice. Among mammalian models used in neuroscience, mice have the widest range of transgenic animals for broad expression of genetically encoded calcium indicators, voltage indicators and reporters of neurotransmitters. Combined with mouse models of neurodegenerative and neuropsychiatric disorders, the mini-mScope should enable studies of mesoscale cortical activity mediating a range of complex behaviors in healthy mice and how these activities may be disrupted in diseased states. The mini-mScope utilizes the CMOS sensor used in the open source ‘miniscope’. The miniscope platform is rapidly evolving and sensors with increased sensitivity and imaging speed, miniaturization and wireless imaging capabilities are being developed. While the SNR of the current mini-mScope is reduced when compared to the conventional epifluorescence macroscope, future versions could incorporate these improved sensors that are optimized for imaging dim voltage indicators.

The mini-mScope performs reflectance measurements at green wavelengths to correct for hemodynamic effects. Multiple wavelength reflectance measurements allow more accurate correction of hemodynamic effects. In future versions, an additional red LED could be incorporated to obtain reflectance measurements at two wavelengths. Alternatively, issues with hemodynamic corrections could be addressed by illuminating GCaMP6f at its isobestic point. The mini-mScope architecture can also be adapted to image red-shifted fluorescent reporters where hemodynamic effects are not prevalent.

The mini-mScope’s FOV is currently limited to the dorsal cortex. Future mini-mScopes could be designed with an expanded FOV to encompass the cerebellar cortex or more lateral regions of the cortex. Finally, mini-mScopes could be designed to incorporate miniaturized amplifiers to integrate chronically implanted recording electrodes for simultaneous mesoscale imaging and deep brain neural recordings or to interface with electrodes incorporated in the See-Shells for electrocorticography.
References
1. Chen, T.-W. et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499, 295–300 (2013).
2. Daigle, T. L. et al. A suite of transgenic driver and reporter mouse lines with enhanced brain-cell-type targeting and functionality. Cell 174, 465–480.e22 (2018).
3. Vanni, M. P. & Murphy, T. H. Mesoscale transcranial spontaneous activity mapping in GCaMP3 transgenic mice reveals extensive reciprocal connections between areas of somatomotor cortex. J. Neurosci. 34, 15931–15946 (2014).
4. Allen, W. E. et al. Global representations of goal-directed behavior in distinct neuronal populations and microcompartments. Neuron 94, 880–890.e8 (2017).
5. Musall, S., Kaufman, M. T., Juavinett, A. L., Gluf, S. & Churchland, A. K. Single-trial neural dynamics are dominated by richly varied movements. Nat. Neurosci. 22, 1677–1686 (2019).
6. Pinto, L. et al. Task-dependent changes in the large-scale dynamics and behavioral correlates in the head-fixed method: stress measurements, functional imaging of mouse cortex. J. Neurosci. Methods 323, 826–829 (IEEE, 2018).
7. Skocek, O. et al. High-speed volumetric imaging of neuronal activity in freely moving rodents. Nat. Methods 15, 429–432 (2018).
8. Ghosh, K. K. et al. Miniaturized integration of a fluorescence microscope. Nat. Commun. 10, 1500 (2019).
9. Murphy, T. H. et al. High-throughput automated head-mounted microscopy for imaging neuronal populations and microcompartments. Nat. Methods 16, 649–657 (2019).
10. Dana, H. et al. Thy1-GCaMP6 transgenic mice for neuronal population imaging in vivo. PLoS ONE 9, e108697 (2014).
11. Ghanbari, L. et al. Cortex-wide neural interfacing via transparent polymer skulls. Nat. Commun. 10, 1500 (2019).
12. Ma, Y. et al. Imaging cortical dynamics in GCaMP transgenic rats with a head-mounted widefield microscope. J. Neurosci. 35, 907–923 (2017).
13. Wekselblatt, J. B., Flister, E. D., Piscopo, D. M. & Niell, C. M. Large-scale imaging of cortical dynamics during sensory perception and behavior. J. Neurophysiol. 115, 2652–2666 (2016).
14. Ghosh, K. K. et al. High-speed volumetric imaging of neuronal activity in freely behaving mice. Nat. Methods 15, 429–432 (2018).
15. Juneau, J., Duret, G., Robinson, J. & Kemere, C. Enhanced image sensor module for head-mounted microscopes. In Proc. Annu. Int. Conf. IEEE Engineering in Medicine and Biology Society (EMBC) 826–829 (IEEE, 2018).
16. Dana, H. et al. Thy1-GCaMP6 transgenic mice for neuronal population imaging in vivo. PLoS ONE 9, 56–60 (2014).
17. Ghosh, K. K. et al. Miniaturized integration of a fluorescence microscope. Nat. Methods 8, 871–876 (2011).
18. Zhao, B. Y. et al. High-speed volumetric imaging of neuronal activity in freely moving rodents. Nat. Methods 15, 429–432 (2018).
19. Scott, B. B. et al. Imaging cortical dynamics in GCaMP transgenic rats with a head-mounted widefield microscope. Neuron 100, 1045–1058.e5 (2018).
20. Namiki, S., Sakamoto, H., Inuma, S., Iino, M. & Hirose, K. Optical glutamate sensor for spatiotemporal analysis of synaptic transmission. Eur. J. Neurosci. 25, 2249–2259 (2007).
21. Marvin, J. S. et al. An optimized fluorescent probe for visualizing glutamate neurotransmission. Nat. Methods 10, 162–170 (2013).
22. Dana, H. et al. Thy1-GCaMP6 transgenic mice for neuronal population imaging in vivo. PLoS ONE 9, e108697 (2014).
Methods
Mini-mScope design, manufacturing and assembly. Design. The mini-mScope was designed using a CAD program (SolidWorks 2018; Dassault Systèmes). The top and bottom housing were CNC-milled by Delrin. The illumination module housing was 3D printed using a desktop stereolithography printer (Form 2; Formlabs) with black poly(methyl methacrylate) (PMMA) resin (catalog no. RS-F2-GBP04; Formlabs). 1.5×2×11 mm3 copper plates were soldered to the large pad on the back of the LEDs to act as heatsinks (Supplementary Fig. 1a). A blue LED (LUXEON Rebel Color Blue 470nm; Digi-Key) and custom diced band-pass excitation filter (450–490 nm, 3×3×11 mm3, ET 470/40; Thorlabs) were installed into an illumination module using ultraviolet (UV)-curable optical glue (AA352 Light Cure Adhesive; LOCTITE) (Supplementary Fig. 1b,c). Each such module was assembled and mounted in the bottom housing of the mini-mScope and the three blue LEDs were serially wired using 29-gauge wires (low-voltage high-temperature black, red wire with fluorinated ethylene polypropylene insulation 29 gauge; McMaster-Carr) (Supplementary Fig. 1d). Two circular black resin was applied to the back of each blue LED to encapsulate the wires for stabilization. A biconvex lens (3-mm diameter, 4.5-mm focal length, 0.33 numerical aperture, achromatic doublet lens; catalog no. 47-721, Edmund Optics) with a numerical aperture of 0.33 and effective focal length of 4.5 mm was gently inserted and press-fitted into the circular slot at the top of the bottom housing, followed by mounting a custom diced band-pass emission filter (500–550 nm, 4×4×11 mm3, ET 525/50m; Chroma) (Supplementary Fig. 1e,f). A green LED (LUXEON Rebel Color Green, 530 nm; Digi-Key) was bonded to the green LED slot of the bottom housing using cyanoacrylate glue (Super Glue Professional; LOCTITE) (Supplementary Fig. 1g). A CMOS sensor (Miniscope CMOS PCB; LabMaker) was fastened to the top housing using M1 thread-forming screws (catalog no. 96817A704; McMaster-Carr). The top housing was slid onto the rectangular shaft of the bottom housing; the focusing was adjusted and then fixed using 316 stainless steel 0–80 screws (catalog no. 91735A262; McMaster-Carr) (Supplementary Fig. 1h). Two circular neodymium magnets (B07CRZZ239; Amazon) were bonded on the bottom surface of the bottom housing with cyanoacrylate glue (Supplementary Fig. 1i).

Wiring. A single coax cable (50Ω 0dB microcoaxial cable; Cooner Wire) was used to connect the CMOS sensor to the main data acquisition (DAQ) board (Miniscope DAQ PCB; LabMaker). For synchronized illumination of alternate frames with blue and green light, the external trigger output from the CMOS sensor was sent to a microcontroller (Teensy 3.5; PJRC). At each odd frame, a microcontroller sent a 3.3-V transistor–transistor logic pulse to a power metal oxide field effect transistor (IRL520 MOSFET; Digi-Key) relay to turn on the three blue LEDs for 20 ms. At each even frame, a second transistor–transistor logic pulse lasting 4 ms was sent to a dedicated MOSFET powering the green LED. The wires powering the LEDs were routed through a circular hole in the top housing and then a commutator (Carousel Commutator 1×20 LED; McMaster-Carr) (Supplementary Fig. 1i). The commutator was rotated using a coaxial silicone rubber-jacketed cable (Cooner Wire) with the blue LEDs in one group and the center of the target was placed onto a 3D-printed inverse mold of the See-Shell contour with markings for eight different test locations in the FOV. A See-Shell implant was pressed onto the inverse mold; target images were taken in each location (Fig. 1e).

see-shell preparation and implantation. The See-Shell implant was assembled using the technique adapted from our previous work. Briefly, the frame of the See-Shell and protective cap were 3D-printed using a desktop stereolithography printer with UV-curable black PMMA resin. A 50-µm thick polyethylene terephthalate film (Melinex 462; Dupont) was bonded to the PMMA frame using quick-setting epoxy (ScotchWeld DP100 Plus Clear; 3M). Two circular neodymium magnets were inserted into the slots on the implant and bottom surface of the protective cap and fixed using cyanoacrylate glue. A 0–80 µm was inserted into the hole in the posterior tab of the implant (Brass Hex Nut, 0–80 thread size; McMaster-Carr).

Surgical implantation. See-Shell implantation. Mice were administered 2 mg kg⁻¹ of sustained-release buprenorphine (Buprenorphine SR-LA; ZooPharm) and 2 mg kg⁻¹ of meloxicam for analgesia and inflammation prevention, respectively. Mice were anesthetized in an induction chamber containing 1–5% isoflurane in pure oxygen. The scalp was shaved and sterilized, followed by the application of sterile surgical preparation (Wound Prep 992100; DowLatex) on the surgical site. The area around the midline was in focus. The adjustment screw was tightened to secure in place. Once focused, the three blue LEDs and green LED were alternately pulsed and their intensities were adjusted by modulating the delivered to the LEDs using each group and the center of the target was placed onto a 3D-printed inverse mold of the See-Shell contour with markings for eight different test locations in the FOV. A See-Shell implant was pressed onto the inverse mold; target images were taken in each location (Fig. 1e).

Illumination profile. To measure the uniformity of illumination, a custom 3D-printed acrylic container was filled with fluorescein dye-infused (10% v/v, catalog no. F2456; Sigma-Aldrich) 3% agar gel. The See-Shell implant was placed on the container such that the entire bottom surface of the See-Shell was uniformly coated with fluorescent gel. The mini-mScope was attached to the See-Shell and single images were acquired. The current delivered to the LEDs was modulated using power supplies in the switching circuit (Supplementary Fig. 2) to eliminate FOV saturation. The captured images were analyzed in MATLAB 2019a (MathWorks) using custom-made code.

LED switching dynamics and LED power stability testing. To test LED stability, the blue LEDs were pulsed at 100 Hz and the output light was measured using a photoreistor (part no. NLS-19MS1; Advanced Photonix). The output voltage of the photoreistor was analyzed in MATLAB to calculate the mean intensity across the FOV (Supplementary Note 2).

In vivo calcium imaging in anesthetized mice. In vivo calcium imaging experiments were performed to compare the mini-mScope imaging capabilities to a conventional macroscope. Mice were lightly anesthetized (0.5–1% isoflurane in pure oxygen) and head-fixed in a stereotax to clean the See-Shell surface of any debris. The mini-mScope was then securely mounted on the implant. The LED was switched on and the position of the top housing relative to the bottom housing was manually adjusted until the area around the midline was in focus. The adjustment screw was tightened to secure in place. Once focused, the three blue LEDs and green LED were alternately pulsed and their intensities were adjusted by modulating the delivered to the LEDs using each power supply (Supplementary Fig. 2).

Focusing and calibration of the mini-mScope. Before every experiment, mice were lightly anesthetized (0.5–1% isoflurane in pure oxygen) and head-fixed in a stereotax to clean the See-Shell surface of any debris. The mini-mScope was then securely mounted on the implant. The LED was switched on and the position of the top housing relative to the bottom housing was manually adjusted until the area around the midline was in focus. The adjustment screw was tightened to secure in place. Once focused, the three blue LEDs and green LED were alternately pulsed and their intensities were adjusted by modulating the delivered to the LEDs using each power supply (Supplementary Fig. 2).

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activity were captured. To evaluate a neural response to sensory stimuli, a 1-s vibrational stimulus was provided to the hind limb using a 3 V DC mini vibration motor (catalog no. A00000464; BestTong) at 100 Hz. For the visual stimulus, a white LED was positioned approximately 2 cm from the left eye of the mouse to cover the fovea in the visual field. Then, 100-ms flashes of light were presented after ensuring stimulus delivery was confined to the left eye.

Open field and social behavior experiments. Mice underwent acclimatization for 3–5 d during which an experimenter handled each mouse for 5–15 min. Mice were fitted with a mini-mScope replica weighing the same as a fully assembled mini-mScope during the handling period. For experimentation, the mini-mScope was fitted onto the mouse and the mouse was quickly transferred to an open field arena. Experimental trials lasted 6 min, including a 2-min period to allow the LEDs to warm up to their maximum intensity. During the social behavior experiments, a C57BL/6 mouse of the same sex was gently introduced into the arena by an experimenter 4 min after the initiation of the trial.

Sleep recording experiment. Mice were habituated for 7 d in the recording setup with the mini-mScope mounted on their head. Each mouse was recorded for 2 sessions, each lasting 2 h. The hippocampal LFP was amplified (x1,000) and filtered (0.1–3000 Hz) using a Grass PS Series AC amplifier (Grass Instrument Company) and was sampled at 1 kHz using a DAQ system (Axon Instruments). A camera (Camera Module V2; catalog no. E305654; Raspberry Pi) was used to record behavior.

Data analysis. Behavior video analysis. Videos of mouse behavior were captured in AVI format using an overhead camera (ELP-US88MP20G-L75; ELP). Mice were tracked using either the Zebrack (version 2.5)1 software in MATLAB or DeepLabCut (version 2.1.5.2)2. Each trial was manually verified to ensure tracking accuracy. A 17-cm square area was defined at the center of the arena as the open field. Movement behavior based on different behavior epochs, 4 researchers manually scored behavior with 1-s precision based on whether the mouse with the mini-mScope was moving, staying still, grooming or rearing and whether the mice engaged in social behavior indicated by touching in ‘contact’ and ‘no contact’ epochs. Scoring data were processed if there was consensus with at least three researchers.

Imaging data preprocessing. Data from the CMOS sensor was captured in AVI format (RGB, 480x752 image size) and contained alternate blue and green channel data. A custom MATLAB script was used to convert the videos from RGB to grayscale and truncated to exclude the first 2 min of LED warmup period at the start of each trial. The resulting video was segmented into individual blue and green channel videos. Blue and green frames were binned using a bilinear spatial binning algorithm in MATLAB. Pixels in each channel were corrected for global illumination fluctuations using a correction algorithm3 and were spatially filtered using a custom weighted spatial filtering algorithm.

ΔF/ΔF calculations. Intensity values from the selected ROIs from the blue and green illumination videos were computed using Fiji v2.1.0/ImageJ v1.53c (ref. 4). Change in fluorescence for both GCaMP and reflectance signals was calculated over a baseline average across the whole time series.

Hemodynamic correction. The green channel ΔF/ΔF traces were filtered using a zero-phase Chebyshev low-pass filter (cutoff frequency of 0.15 Hz). The blue ΔF/ΔF and filtered green ΔF/ΔF traces were used to perform hemodynamic correction as described previously5. The hemodynamics-corrected traces were filtered using a zero-order phase Chebyshev band-pass filter (cutoff frequencies of 0.1 and 5 Hz). Then, a baseline signal was calculated by averaging all the frames and the fluorescence changes were quantified as ΔF/ΔF×100, where F is the filtered signal. To reduce spatial noise, images were filtered by a Gaussian kernel (5x5 px, σ = 1).

State scoring. Behavioral states were scored visually using hippocampal LFP and movement signal in 10-s epochs. Movement signals were calculated using an algorithm described previously6. Active wakefulness was characterized by theta hippocampal activity and high movements. Quiet wakefulness was characterized by theta hippocampal activity and minimum movement. Non-REM sleep was characterized by large irregular activity in the hippocampus and no movement. REM sleep was characterized by theta hippocampal activity and no movement.

Correlation analysis. A uniform meshgrid with an approximate 1.2-mm distance between its points was laid on the FOV and the signal at each ROI (0.2 mm²) was calculated. Quiet wakefulness, non-REM and REM sleep were scored for each recording based on the above criteria and PCCs were calculated between each ROI during quiet wakefulness, non-REM and REM sleep. For comparison in Fig. 4f, correlation matrices were averaged across all ROIs.

Statistics and reproducibility. Raw cortical images, similar to the representative images in Fig. 1c,d were taken in all mice and can be reproduced throughout the figures in the manuscript. Sample sizes for all numerical data in the graphs have a minimum n = 3 and statistical tests were performed with a minimum n = 5.

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

Data availability. Data containing videos, pseudocolor maps and images are available upon request from the authors due to the large file sizes. The Allen Brain Atlas was used as an anatomical reference for data analysis in this study (http://www.brain-map.org). All CAD files for manufacturing the mini-mScope are available with this article as Supplementary Data 2. Source data are provided with this paper.

Code availability. All custom code is available on Github at https://github.com/bsbrl mini-mScope.

References
43. Ghanbari, L. et al. Craniobot: a computer numerical controlled robot for cranial microsurgery. Sci. Rep., 9, 1023 (2019).
44. Ryves, M. L. et al. Assembly and operation of an open-source, computer numerical controlled (CNC) robot for performing cranial microsurgical procedures. Nat. Protoc., 15, 1499–1503 (2020).
45. Silasi, G., Xiao, D., Vanni, M. P., Chen, A. C., & Murphy, T. H. Intact skull chronic windows for mesoscopic wide-field imaging in awake mice. J. Neurosci. Methods 267, 141–149 (2016).
46. Pinheiro-da-Silva, J., Silva, P. F., Nogueira, M. B. & Luchiarri, A. C. Sleep deprivation effects on object discrimination task in zebrafish (Danio rerio). Anim. Cogn. 20, 159–169 (2017).
47. Mathis, A. et al. DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. Nat. Neurosci. 21, 1281–1289 (2018).
48. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
49. Lau, C. et al. Exploration and visualization of gene expression with neuroanatomy in the adult mouse brain. BMC Bioinformatics 9, 153 (2008).
50. Singh, S., Bermudez-Contreras, E., Nazari, M., Sutherland, R. J. & Mohajerani, M. H. Low-cost solution for rodent home-cage behaviour monitoring. PLoS ONE 14, e0220751 (2019).
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Author contributions
M.L.R., L.G., M.L., D.A.S., L.G., G.W.J. and S.B.K. designed and engineered the mini-mScope. M.L.R., D.A.S., J.D., Z.S.N., O.H., L.G. and S.B.K. designed and executed the experiments. M.L.R., D.A.S., S.L., O.H., V.R. and S.B.K. analyzed the data. M.L.R., D.A.S., S.L., V.R., J.D., M.L. and S.B.K. wrote the manuscript. M.N. and M.H.M. designed and executed the glutamate imaging experiments, analyzed the data and assisted with manuscript writing.

Competing interests
The authors declare no competing interests.

Additional information
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  Give P values as exact values whenever suitable.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Miniscope Control Software v3 was used to program the CMOS sensor to capture fluorescent cortical data during sensory evoked responses, free behavior, social behavior, and glutamate imaging. Spontaneous calcium activity under light isoflurane anesthesia was captured using HCImageLive v4.5.1.3. Electrophysiology data was captured using pClamp 10 software suite.

Data analysis
Analysis for sensory evoked stimuli, free behavior, social behavior, and glutamate imaging were performed in MATLAB 2019a (Mathworks Inc.) using custom code. Motion correction and blood vessel analysis were performed in Fiji 21.0 (ImageJ 1.53c). Statistical analyses were performed in MATLAB 2019a.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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Source data for plots in figures (except for videos, pseudocolor maps, and images) are provided with this paper as Supplementary datasets. Data containing videos, pseudocolor maps, and images are available upon request of the authors due to large file sizes. The Allen Brain Atlas was used as an anatomical reference for data analysis in this study (http://www.brain-map.org).
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No sample size calculation was made. Our goal was to demonstrate a new technology for widefield imaging. All experiments were performed at a minimum of 3 mice, and when statistical testing was used, the minimum sample size was 5.

Data exclusions
No data was excluded.

Replication
In this study the main biological units are mice. All experiments were performed on multiple mice (n = 3 or greater) and were reproducible. Some GCaMP6f mice were used in multiple experiments, e.g., social behavior as well as open field experiments.

Randomization
We did not perform any randomization, because the experiments were performed to demonstrate the efficacy of the methodology presented, and not to make statistical claims about a biological phenomena or mechanism.

Blinding
Experiments were performed to either characterize the performance of the mini-mScope or demonstrate its imaging capabilities. We did not have a specific hypothesis about specific conditions and how this would affect the performance of the device, therefore we did not find it necessary to design experiments with blinding.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology and archaeology |
| ☑ ☐| Animals and other organisms |
| ☑ ☐| Human research participants |
| ☑   | Clinical data         |
| ☑   | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChiP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

Animals and other organisms

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

Laboratory animals
C57BL/6j, Thy1-GCaMP6f, and Emx-CaMKII-Ai85 mice were used in the experiments. Both male and female mice were used, aged 8-30 weeks. Mice were housed in a 14 hour light / 10 hour dark cycle.

Wild animals
This study did not involve wild animals

Field-collected samples
This study did not involve samples collected from the field

Ethics oversight
All animal experiments were conducted in accordance with protocol approved by the University of Minnesota’s Institutional Animal Care and Use Committee (IACUC) and the University of Lethbridge’s Animal Care Committee.

Note that full information on the approval of the study protocol must also be provided in the manuscript.