

Figure S1. Detailed technical adaptations for dual-mount *in vivo* pan-cortical imaging with 2p-RAM mesoscope. **a)** Photographs of widefield through-skull (top) and 2-photon cranial window (bottom) states of side mount preparation. Bregma (β) and lambda (λ) skull landmarks used in multimodal mapping alignment to common coordinate framework (CCF) map are indicated (top), along with their corresponding positions relative to cortical blood vessels visible through cranial window (bottom). Note that all visible tissue inside the perimeter of the headpost in the lower image is seen through curved, 210 μm thick borosilicate Schott D263 glass. **b)** Custom aluminum support arms for use with both dorsal mount and side mount preparations. Fixed arm (top) has flat proximal angle at bend near dual-screw headpost mounting slot (right red arrow), and can be mounted on a Thorlabs 1-inch diameter post mounted to a rotating ball-joint base

(SL20, see suppl materials) to achieve leftward 22.5-degree rotation for side mount (not shown). Flexible positioning on wheel is possible due to $\frac{1}{4}$ "-20 bolt accepting slot that allows support posts to be positioned closer or farther from headpost mounting slot (left red arrow). Custom adjustable arm (bottom) has rotating brass shaft attached to ball-and-socket joint with hexagonal tightening ring (top, long black arrow), stabilized by dual set screws on both ends (bottom, two short black arrows), at a bend proximal to the headpost mounting slot. This allows for rapid, fixable on-rig fine adjustment of the mounting angle in all directions, and eliminates the need for a Thorlabs rotating ball-joint base so that 1" diameter vertical posts can be used (instead of $\frac{1}{2}$ " diameter, which we used at first), thus enhancing overall stability for imaging. **c)** Examples of headpost mounting for dorsal mount (top, with cranial window) and side mount (bottom, headpost only). Both headposts are attached with two 82 degree countersunk 2-56 thread 1/4 " stainless steel screws (McMaster-Carr, see Suppl Materials) and positioned in a custom machined slot within the support arm for enhanced stability. The headpost arm can easily be held within the support arm slot by thumb prior to screw affixation during mounting. **d)** Mounting of 3D printed light shields and application of objective to water meniscus. Flat shields (top left, bottom left) are attached with 1:1 rapid curing Sylgard (top right; 170 Fast Cure encapsulant, see Suppl Materials) to the perimeter of the headpost. Stable positioning during curing is enabled by attachment of dual 3-pronged lab clamps attached to left and right support arms with quick ties (not shown). Then, a tip-clipped transfer pipette is used to add deionized or Millipure water to the window, with the Sylgard acting as a dam, until the objective can be lowered far enough to raise a meniscus. Additional water can then be added from either side of the preparation under the objective. **e)** Side view 3D rendering (left, from AutoDesk Inventor; L = left, R = right, A = anterior, P = posterior, center-line = dashed-line) and top view photograph with cranial window placed on paraformaldehyde-fixed brain, for dorsal mount preparation. **f)** Same as in e), but for side mount preparation. **g)** Cumulative success rate “survivorship” diagram for dorsal and side mount preparations (note: survival rate is higher, but some preparations are not fully imageable, some mice fail to acquire the task, etc), loosely based on empirical data from mice both used to develop the preparations, those used in this study, and those used in pilot experiments for 2-alternative forced choice (2-AFC) behavior. Note that the standard full timeline involves around 100 days from headpost implantation to experiment completion. In our experience, successful cranial windows last between 100-150 days, or in some rare cases up to 300 days.

Movie S1. Dorsal mount: Upper left, 3D printed titanium headpost for dorsal mount (left; i.materialise.com and sculpeo.com) and accompanying cranial window for dorsal mount (labmaker.org, or TLCInternational.com and glaswerk.com), shown in top and side orthogonal projection, followed by isometric projection (AutoDesk Inventor, Adobe Acrobat Pro 3D viewer). Upper right, 3D printed plastic (PLA) light-shields or “woks”, same views as upper left. Horizontal light shield (left) fits onto perimeter of dorsal mount headpost and is attached with Sylgard 170 Fast Cure silicone elastomer, and vertical light shield fits onto vertical perimeter ridge of horizontal light shield and is held by gravity. Bottom, simultaneous and temporally aligned high-resolution videography from three points-of-view of a mouse under spontaneously behaving conditions (shown at 3x speed or 90 Hz; left and right camera are GigE Teledyne Dalsa M2050 cameras, and posterior camera is FLIR grasshopper USB3 a camera). Example pose tracking labeling by DeepLabCut (Mathis et al, 2018) is shown (right camera). Side mount: same as in dorsal mount video, but for side mount hardware. Note that the mouse’s headpost is retained by a single fixed support arm, rotated 22.5 degrees to the left, whereas the mouse in the dorsal mount example is held by dual orthogonally positioned fixed support arms.

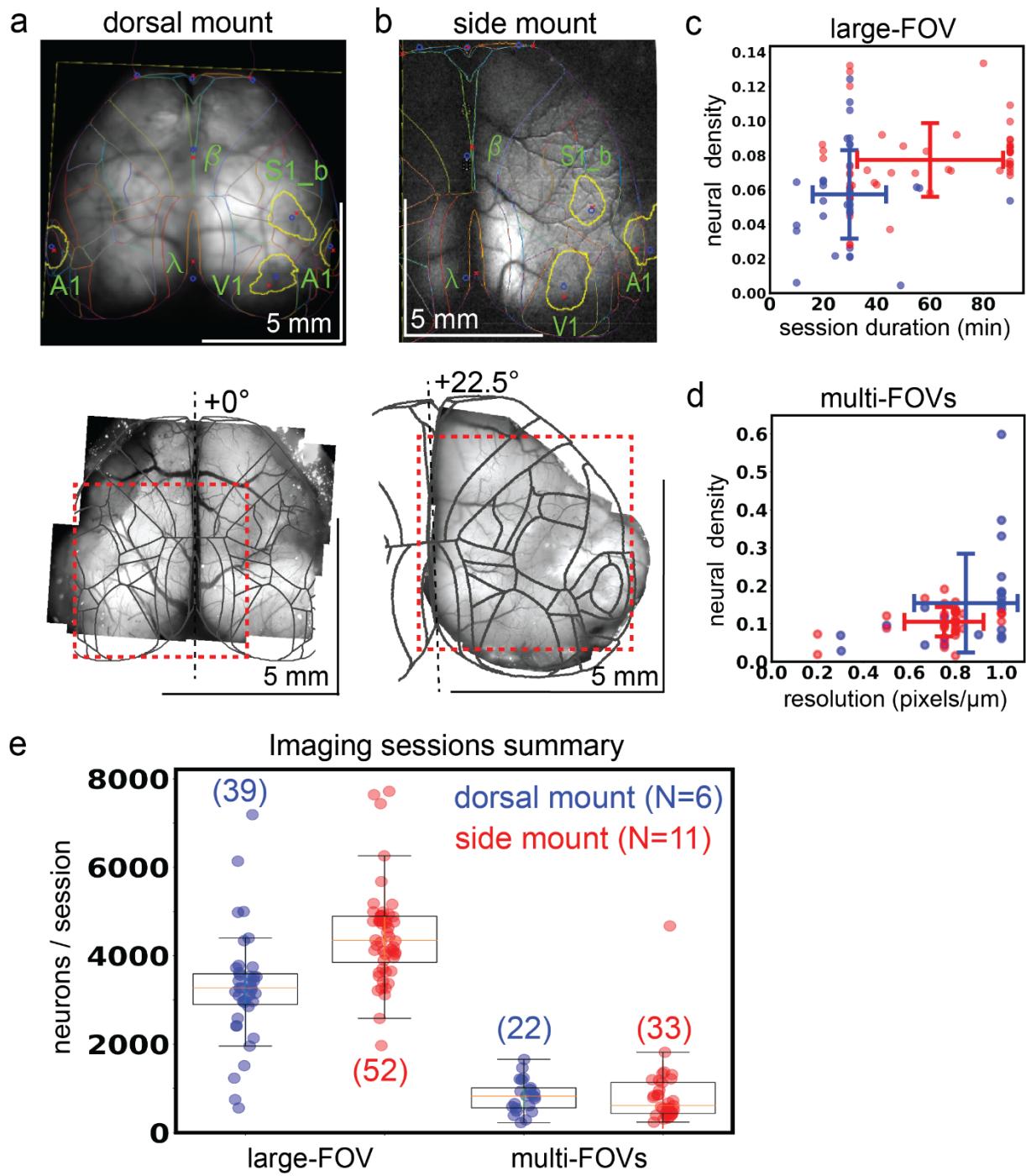


Figure S2. Widefield-imaging based dual-mount multimodal CCF alignment, ROI detection, and areal assignment of GCaMP6s⁺ excitatory neurons. **a)** Example MMM to CCF alignment for dorsal mount, shown projected onto a through-the-skull vasculature image and including skull landmarks (top). The series of user-selected points used for alignment of the widefield image onto the CCF map are shown as blue points, with corresponding red points showing the actual mapping locations used by the affine transformation. 2p-RAM pseudo-widefield vasculature

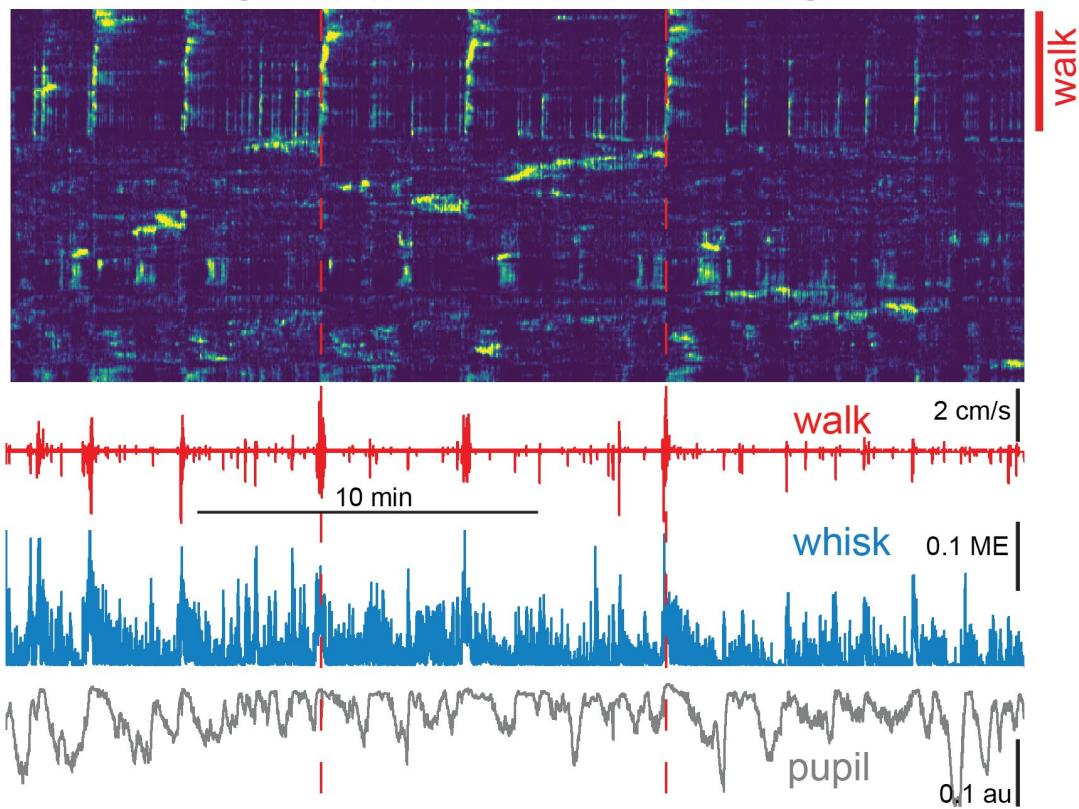
montage from the same mouse, with boundaries of example 2-photon imaging session FOV shown as red dashed line (bottom). View angle relative to orthogonal indicated at top of dashed black line indicating mid-line. **b)** Same as in a), but for example side mount preparation. **c)** Density of Suite2p-detected ROIs (density of 1 equal to one neuron per $25 \times 25 \mu\text{m}$ square; total large FOV area generally $5000 \times 4620 \mu\text{m}$) for large field of view (FOV) 2-photon imaging sessions as a function of session duration for dorsal mount (blue) and side mount (red) preparations. **d)** Neural density as a function of imaging resolution for 2-photon imaging sessions where multiple smaller FOVs were imaged pseudo-simultaneously. Same density calculation used as in c), for multi-FOV sessions where the dimensions of each small FOV are generally between 660×660 and $1320 \times 1320 \mu\text{m}$. Here, a resolution of 0.2 pixels / μm would correspond to 5 μm per pixel resolution in both x and y dimensions (i.e. $\frac{1}{5} = 0.2$). **e)** Summary statistics for all 2-photon imaging sessions in the current study that passed quality control for all data modalities (i.e. MMM, 2-photon, behavior video, etc). The number of neurons per 2-photon imaging session is shown for both large- (two columns at left) and small- (two columns at right) FOV sessions, split by mount type (dorsal mount in blue, side mount in red). Numbers in parentheses indicate total session counts ("S"), "N" indicates number of mice with each mount type. FOV = field of view, V1 = primary visual cortex, A1 = primary auditory cortex, S1_b = primary somatosensory barrel cortex, β = bregma, λ = lambda.

Movie S2, Dorsal mount multimodal mapping. Example widefield (1photon) imaging GCaMP6s fluorescence responses for visual and whisker multimodal mapping sessions. Visual: top right, full field, left-side isoluminant Gaussian noise stationary grating patches (vertical and horizontal stationary grating patches; 0.16 cpd, 30 deg; Michaiel et al, 2019) presented to elicit visual response in right cortex, with small upper left-corner alternating white/black box positioned under photodiode to record precise stimulus presentation times. Top left, pixelwise dF/F response of the entire image for a single trial, recorded at 50 Hz and shown at 0.5x speed. The baseline was calculated as the median of a 1 s period leading up to the stimulus onset. The dashed white circle indicates the putative primary visual cortex (right V1). A = anterior, L = left, R = right, P = posterior, dF/F = change in fluorescence divided by baseline fluorescence; midline extends vertically near the center of frame from bottom to top edge roughly between the "A" and "P": labels. Bottom, trace of mean dF/F for all pixels inside dashed white circle (mask), expressed as percent change. Vertical black line indicates stimulus onset. The visual stimulus is present through the end of the epoch shown. Upper left, overlay: mean of 33 dF/F responses (mean of 1 second after stimulus onset minus mean of 1 second leading up to stimulus onset) in

a single dorsal mount session under 2-3 % isoflurane anesthesia. ITI = inter-trial interval, measured from beginning of one stimulus to beginning of the next stimulus. Whisker: same as in visual example video, with same mouse on same day, except with 5 Hz, 100 ms duration forward swipes with custom 3D printed plastic (PLA) whisker-deflector, as indicated by vertical deflections in stimulus trace, mid-right. Example video of mouse shown from a different session than dF/F data, because mouse face video was typically not recorded during multimodal alignment sessions. Red S1b (and dashed line) = right primary whisker barrel cortex. A = anterior, P = posterior, L = left, R = right, V1 = primary visual cortex.

a

Session 1: online z motion correction OFF



b

Session 2: online z motion correction ON

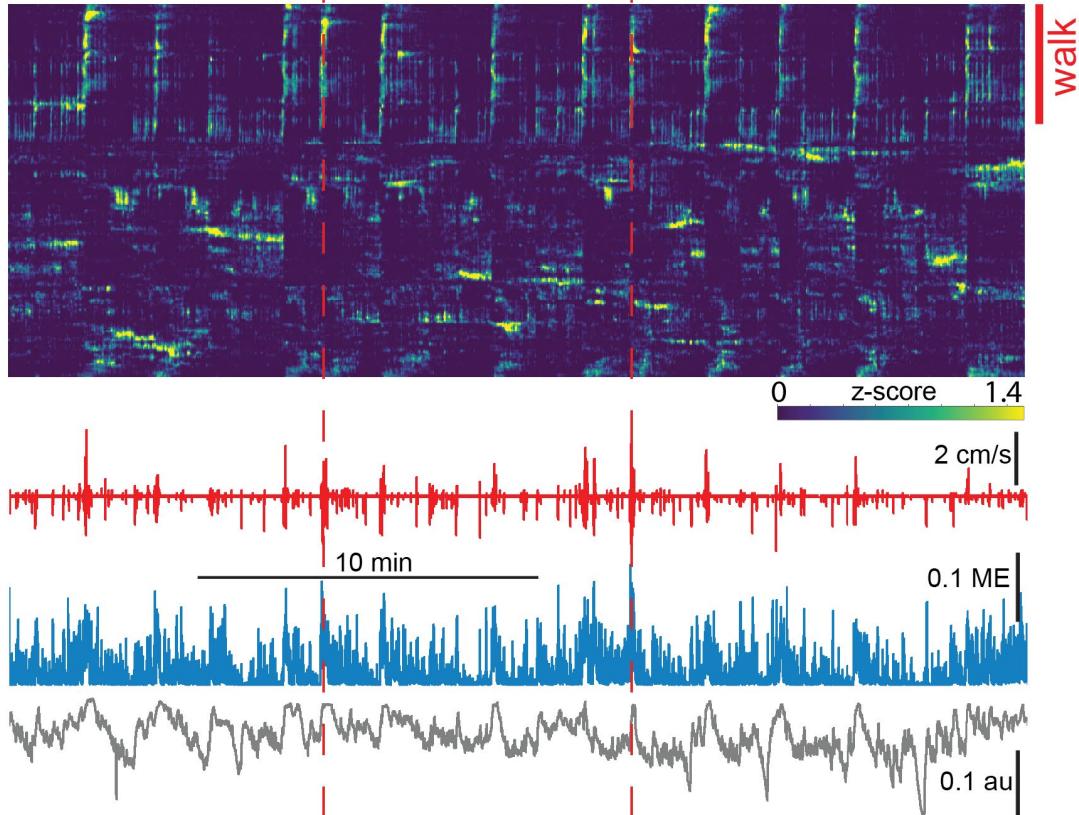


Figure S3. Comparison of neurobehavioral alignment in back-to-back mesoscope imaging sessions either without (**a**, “OFF”) and with (**b**, “ON”) GPU-enabled online z-motion correction enabled. **a)** Neural and behavioral data are shown temporally aligned for the first session. Rastermap sorted neural activity is shown at top, with a blue/green/yellow (min/mid/max) heatmap applied to activity from each individual neuron z-scored separately. Walk speed (red), whisker motion energy (blue), and pupil diameter (gray) are shown below. Red dashed vertical lines indicate times of two major walking bouts for the purpose of alignment visualization. Note that roughly the top ~30% of neurons show strong activity time-locked to the identified walking bouts (red vertical line at right, labeled “walk”). **b)** Same as in a), except for a 2nd consecutive session in the same mouse. The only change was that, for this session, online GPU-enabled z-motion correction was enabled (“ON”) following acquisition of a $60 \times 1 \mu\text{m}$ step anatomical z-stack for frame-by-frame comparison and adjustment (ScanImage/Vidrio). Note the similar percentage of cells aligned with each walking bout (top ~30% in Rastermap sorts in both a) and b) (indicated by red vertical line at right labeled “walk”), and the overall qualitative similarity of Rastermap clustering and activity motifs compared to that of the immediately prior session shown in a), despite the fact that these back-to-back sessions contained different detailed patterns of spontaneous activity. ME=motion energy, au=arbitrary units, GPU = graphics processing unit.

Movie S3, Side mount multimodal mapping. Visual, Whisker, and Auditory, as in Movie S2 but for side mount preparation. Same mouse in Auditory and Whisker examples, different mouse in Visual example. 1.5-3% isoflurane anesthesia was used in all 3 sessions. Auditory: 1 s tone cloud with tones between 2 and 40 kHz presented for 0.5 s starting at black vertical dashed line (bottom). Sonogram display from Spike2 (CED) shows individual tones as horizontal green lines, where y-axis is sound frequency (~0 – 25 kHz) and x-axis is time (0 to 1 s). Movies shown at 0.25x speed. As in other example videos, the mouse shown is from a different session, but is exposed to the same stimulus at the indicated time. The mouse shown is from a different session type when videography was enabled, to show the normal response of the mouse to the stimulus. ml = midline, A1 = primary auditory cortex, A = anterior, L = left, R = right, P = posterior, dF/F = change in fluorescence divided by baseline fluorescence.

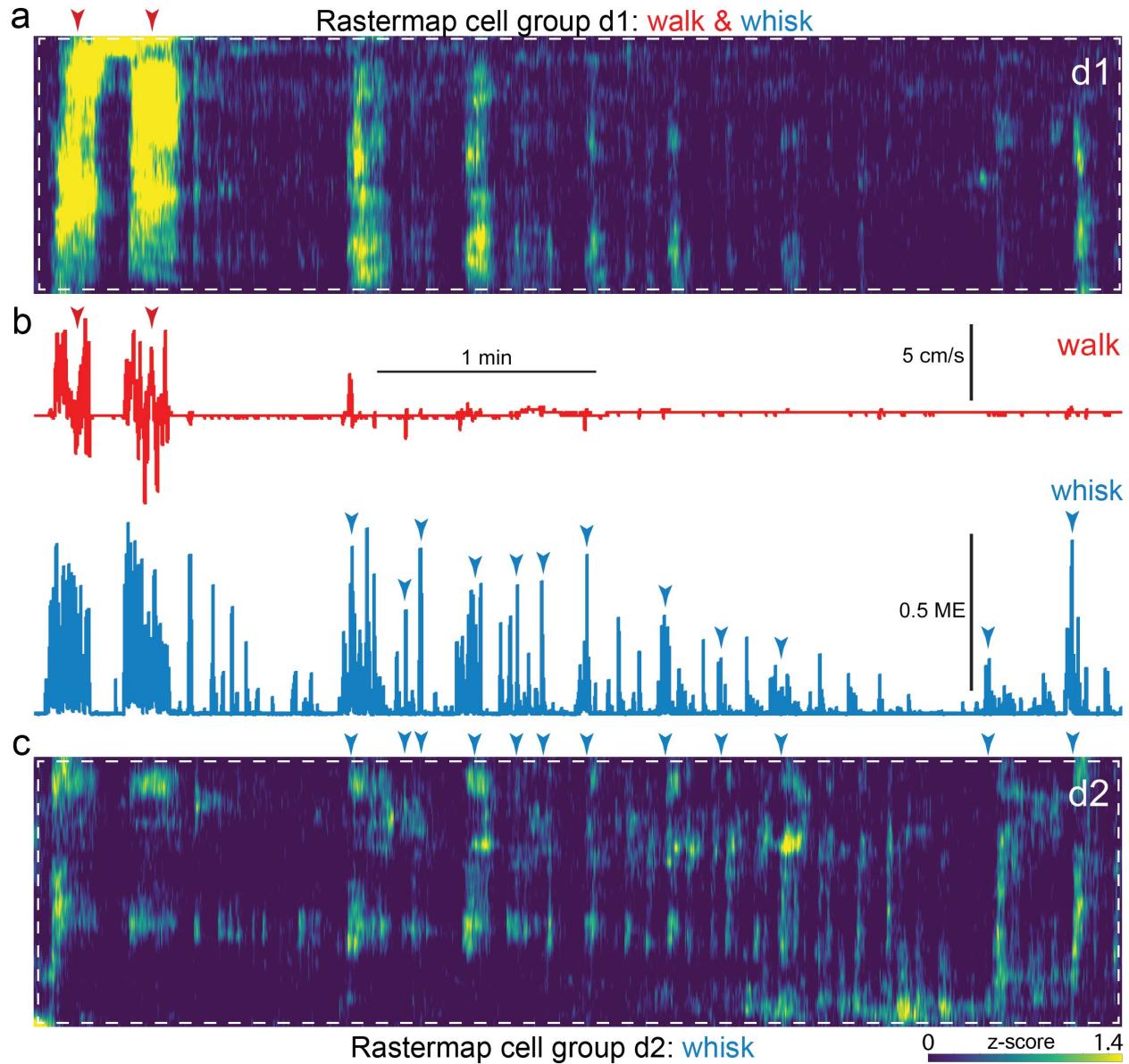


Figure S4. Expanded insets of Fig 4 (d1), or walk and whisk Rastermap group (**a**, white dashed box) and Fig 4 (d2), or whisk Rastermap group (**c**, white dashed box), shown aligned to walk speed and whisker motion energy (**b**, top, red, and bottom, blue, respectively). A blue/green/yellow (low/mid/high) heat map was applied to individually z-scored neural activity (**a**), as indicated by scale bar (bottom right). Neural data is shown vertically temporally aligned to behavioral arousal and movement data (**b**). Walk speed (red) and whisker motion energy (blue) are shown for this example dorsal mount session. Red and blue arrowheads indicate temporal alignment of walk and whisk bouts, respectively, between neural and behavioral data. ME=motion energy.

Movie S4. Dorsal mount. Titanium 3D printed headpost shown from above, with paraformaldehyde-fixed mouse brain shown beneath custom cranial window, colored Allen CCF outlines, and an example 5 x 4.62 mm mesoscope FOV corresponding to data in this video shown inside an inset box indicated by a white dashed line. Next segment, left, full 5 x 4.62 mm ScanImage (Vidrio) rendered mesoscope 2-photon field of view, rotated and flipped so that the top corresponds to the front of the mouse and the left corresponds to the left hemisphere of the cerebral cortex. Black horizontal joining lines indicate the “seams” between adjacent ROIs where they are joined by rendering. The resonant scanner moves along the short axis of each rectangle, perpendicular to the “seams”, and mechanical galvanometers move along the long axis, parallel to the “seams”. Midline vertically oriented (i.e. from posterior, at bottom, to anterior, at top) blood vessel (sinus) prominent at center of frame. Movie (100 s duration shown) acquired with unidirectional scanning at 1.62 Hz, smoothed with a running average of 3 frames, and shown at 3x playback rate. Center top, expanded inset from white dashed box (left), shown synchronized with larger video. Bottom center, high-resolution left face videography of mouse from this session, synchronized with 2-photon video, rasterized neural data (upper right), and Spike2-recorded (CED) behavioral data (whisking motion energy in blue, pupil diameter in gray, and walk speed in red). Neural data was sorted in Suite2p (Stringer et al, 2019) with Rastermap (top), or by its first (middle) or second (bottom) principal component of activity over the entire session, and is displayed as z-scored (normalized) dF/F neuropil subtracted activity indexed to a single color-map lookup table with yellow as maximum, green as intermediate, and blue/purple as minimum activity level (i.e. GCaMP6s fluorescence; same lookup table scale as in Fig. 4a). Red arrowheads indicate co-alignment of transient arousal increases accompanied by walking, whisking, and pupil dilation, with diverse changes in neural activity across rastermap, PC1, and PC2 sorted ensembles.

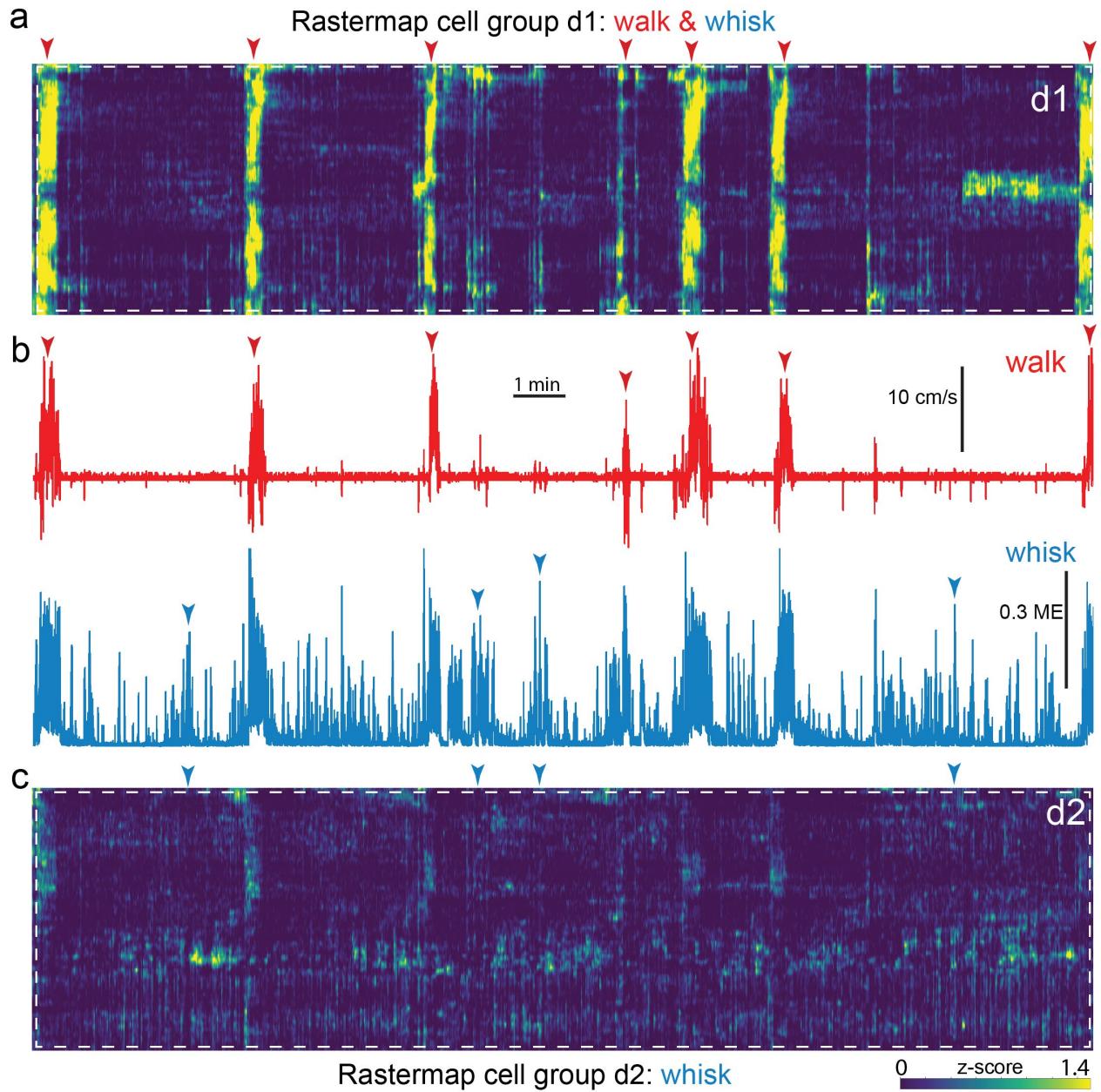


Figure S5. Expanded insets of Fig 5 (d1), referred to here as the walk and whisk Rastermap group (**a**, white dashed box) and Fig 5 (d2), referred to here as the whisk Rastermap group (**c**, white dashed box), shown aligned to walk speed and whisker motion energy (**b**, top, red, and bottom, blue, respectively). A blue/green/yellow (low/mid/high) heat map was applied to individually z-scored neural activity (**a**), as indicated by scale bar (bottom right). Neural data is shown temporally aligned to behavioral arousal and movement data (**b**). Walk speed (red) and whisker motion energy (blue) are shown for this example side mount session. Red and blue arrowheads indicate temporal alignment of walk and whisk bouts between neural and behavioral data. ME=motion energy.

Movie S5. Example 2 dimensional field of view (FOV) rendered movie and insert from an example session where factorial hidden Markov modeling showed near global synchrony of PC1 loading during walking bouts in a side mount mouse. A 5 x 5 mm full FOV movie (~10x real-time) is shown at top left, with a 1 x 1 mm inset (white dashed box) expanded and shown at bottom left. Three example Suite2p-extracted neuron ROIs (1, 2, and 3) and a blood vessel ROI (bv) are indicated with white dashed circles. Gray traces at right indicate mean pixel fluorescence intensity of all pixels within each of these ROIs over ~450 2-photon movie frames. Rasterized rendering at top right indicates PC1 loading (blue/green/yellow=low/medium/high) across all CCF areas contained in the 2p movie (one per row). The red horizontal lines at the end of the shown epoch are a graphical rendering artifact that was not removed. White/gray/black rows indicate the current state of each of four hidden factors (i.e. state 0 = white, state 1 = gray, and state 2 = black) in a factorial hidden Markov model (fHMM) fit to the first 15 PCs of global neural data, the blue row indicates walk speed, the next, split blue row indicates left and right whisker pad motion energy, and the red, bottom row indicates pupil diameter. Actual behavioral arousal and movement primitives are shown as traces below (walk=red, whisk=blue, pupil = gray). Note that walk-related activity changes are correlated with independent fluctuations across the three example neurons, and furthermore that blood vessel fluctuations are not small and not movement-locked, consistent with the idea that neurobehavioral activity alignment in this case, even with detected transient global synchrony across the first PC of activity, is not driven by brain movement artifact.

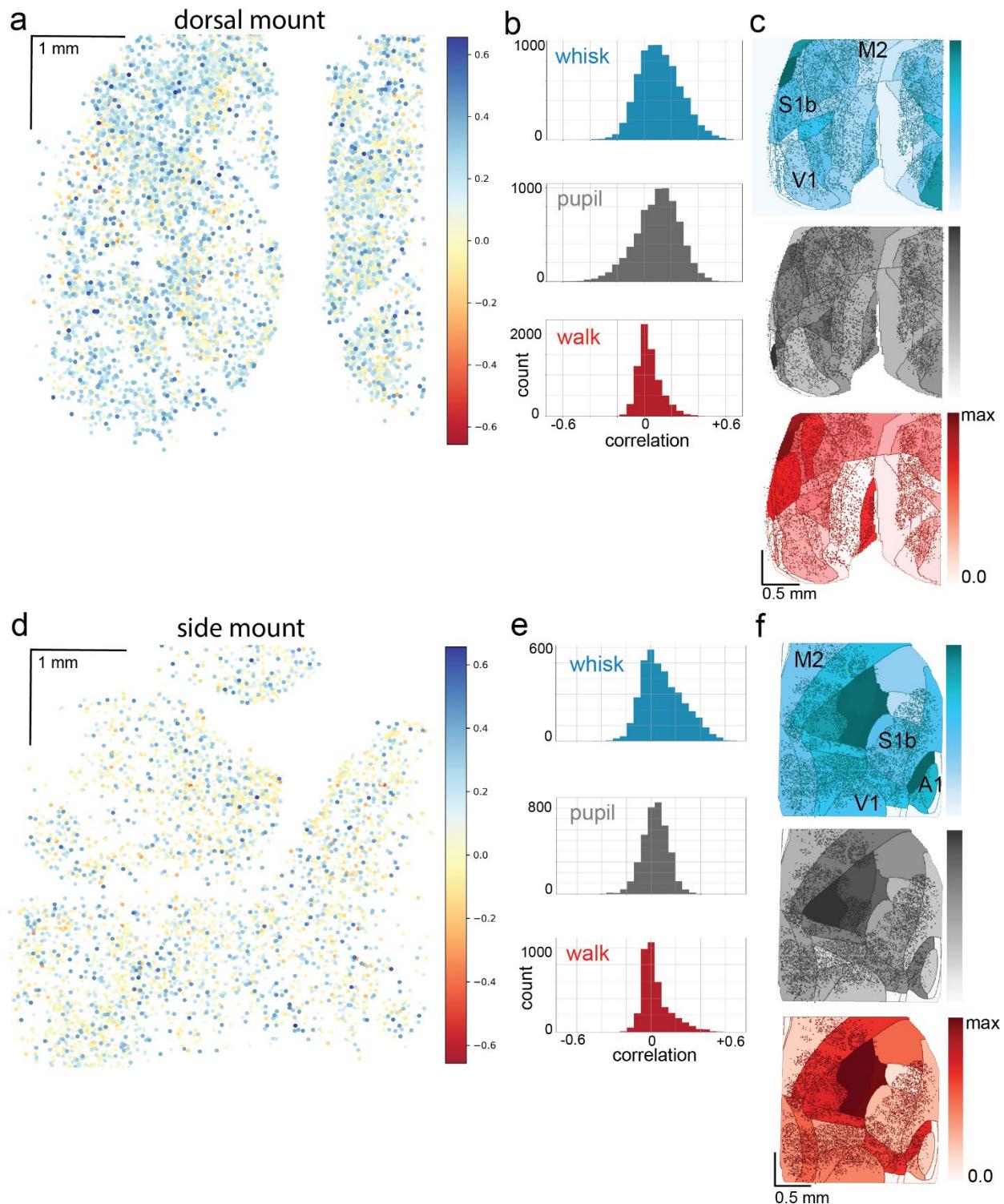


Figure S6. Heterogeneity of arousal and movement correlations with neural activity in the dorsal and side mount preparations. **a)** Color coded correlations of dF/F with whisker motion energy in individual neurons of the dorsal preparation in a different example session from the same mouse as in Fig. 4, with large positive correlations shown in blue, large negative correlations

shown in red, and small correlations shown in white/light blue/light red. **b)** Histograms of whisk ME, pupil diameter, and walk speed (cm/s) for the same example session as in a). **c)** Normalized mean of mean correlations of neural activity with behavioral arousal primitives of whisker motion energy (blue; mean: MIN = 0.00, MAX = 0.05; standard deviation (STD): MIN = 0.002, MAX = 0.045), pupil diameter (gray; mean: MIN = 0.00, MAX = 0.06; STD: MIN = 0.004, MAX = 0.038), and walk speed (red; mean: MIN = 0.00, MAX = 0.02; STD: MIN = 0.004, MAX = 0.018) for each CCF area across eight sessions from the same dorsal mount mouse shown in Fig. 4. Mean whisker motion energy correlations with neural activity (dF/F) across these 8 sessions from the same mouse were significantly more than zero ($p < 0.05$, median $t(7) = 3.0$, single-sample t-test; python: `scipy.stats.ttest_1samp`) for 24 of the 27 CCF areas with at least 20 neurons present. The areas with mean correlations not significantly larger than zero were left SSp-m, SSp-n, and SSp-un. Mean pupil diameter correlations with neural activity (dF/F) were significantly more than zero ($p < 0.05$, median $t(7) = 1.3$, single-sample t-test) for 8 of the 27 CCF areas, including left VISp, VISam, VISpm, MOp, and SSp-II, SSp-ul, and right VISp and SSp-ul. Mean walk speed correlations with neural activity (dF/F) were not significantly more than zero ($p < 0.05$, median $t(7) = 1.0$, single-sample t-test) in any of the 27 CCF areas. **d)** Color coded correlations (same lookup table as in a) of dF/F with whisker motion energy in individual neurons of the side mount preparation in an example session from the same mouse as in Figs 5 and 6. **e)** Histograms of whisk ME, pupil diameter, and walk speed (cm/s) for the same example session as in d). **f)** Normalized mean of mean correlations of neural activity with behavioral arousal primitives of whisker motion energy (blue; mean: MIN = 0.00, MAX = 0.08; standard deviation (STD): MIN = 0.000, MAX = 0.043), pupil diameter (gray; mean: MIN = 0.00, MAX = 0.02; STD: MIN = 0.000, MAX = 0.034), and walk speed (red; mean: MIN = 0.00, MAX = 0.01; STD: MIN = 0.000, MAX = 0.029) for each CCF area across eight sessions from the same side mount mouse shown in Figs 5 and 6. Mean whisker motion energy correlations with neural activity (dF/F) across these 8 sessions from the same mouse were significantly more than zero ($p < 0.05$, median $t(7) = 5.3$, single-sample t-test; python: `scipy.stats.ttest_1samp`) for 23 of the 23 CCF areas with at least 20 neurons present. Mean pupil diameter correlations with neural activity (dF/F) were significantly more than zero ($p < 0.05$, median $t(7) = 1.2$, single-sample t-test) for the right VISI and right TEa areas only. Mean walk speed correlations with neural activity (dF/F) were not significantly more than zero ($p < 0.05$, median $t(7) = 1.1$, single-sample t-test) in any of the 23 CCF areas.

Movie S6. Side mount. Same as in Movie S4, but for an example session from a side mount mouse instead of a dorsal mount mouse. Data from 100 s contiguous segment of single session, played at 3x original speed (3.06 Hz 2-photon acquisition, bidirectional scanning with 3-frame running average applied). Right cortex is shown (5 x 4.62 mm total at 5 micrometer resolution per pixel in x and y), with anterior at front (top), auditory cortex at bottom right, and midline at left edge of frame. Behavioral videography shown is taken from the right side and shows the entire front end of the mouse including torso, paws, ears and face. Time scaling is the same as in Movie S4.

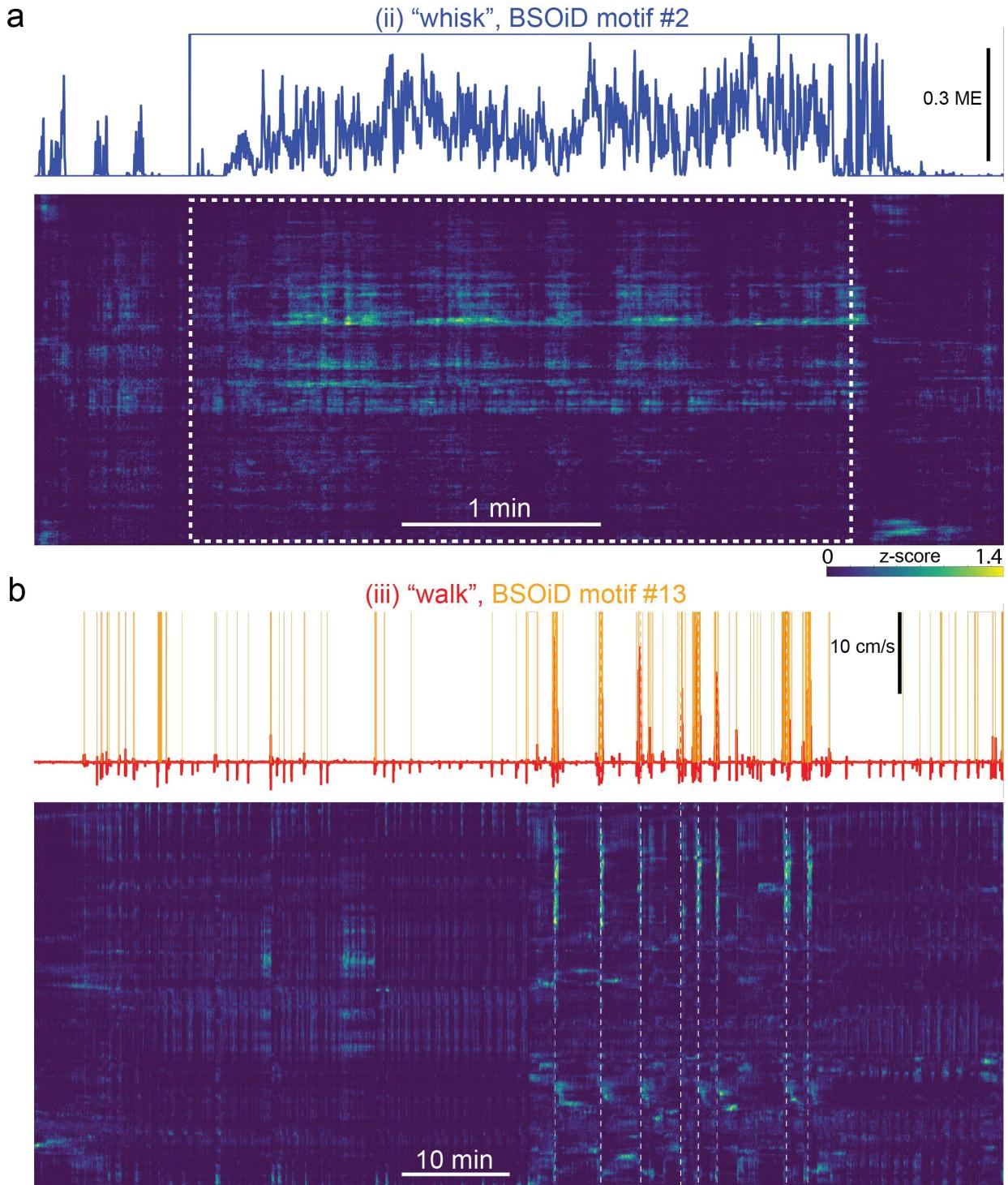


Figure S7. Example expanded alignment of high-level behaviors and primary corresponding BSOiD motifs, arousal measures, and Rastermap sorted neural data from side mount preparation, same example session as in Figs 5, 6. **a)** Inset (ii) from Fig 6a, an example of whisk behavior. Whisker motion energy (solid blue line, top) shown aligned to BSOiD motif #2

(one-hot encoded, solid blue square-wave line; up=1 (ON), down=0 (OFF), and Rastermap sorted neural activity (z-scored for each neuron; blue=min, green=intermediate, yellow=high) for this example session. Number of neurons=5678. **b**) Same session and display as in a), but for inset (iii) walk and BSOiD motif #13 (yellow) from Fig 6a. White vertical dashed lines are aligned with major walking bouts. ME=motion energy.

Movie S7. Example simultaneous dual color mesoscope acquisition shows independence of activity-driven Ca^{2+} transients from movement artifacts. **a), c)** Simultaneous dual color acquisition of activity-independent EYFP (top) and activity-dependent RCaMP (bottom). Dashed white circles show ROIs whose mean pixel intensities are shown in b), d). **b)** Mean raw pixel intensity for the 2 ROIs shown in a). The blue arrow indicates an example of blanking during 473 nm optogenetic stimulation (i.e. PMT is shuttered during 473 nm laser stimulation, so that section of trace is removed because it does not reflect actual fluorescence activity from the mouse cortex), and the red arrow shows an example of walking-induced movement of the imaged FOV. **d)** Same as in b), but for 3 ROIs shown in c). Note that the magnitude of the movement artifact related transient in the Ca^{2+} indicator fluorescence (i.e. the red channel, PMT2) is relatively unchanged relative to adjacent peaks in ROI1, an identified neuron, and is very small or nonexistent in other neuropil and blood vessel ROIs, and in the yellow anatomical channel (PMT1). PMT=photomultiplier tube.

Protocol I.

Overall workflow

1. Headpost implantation in mice between 8 and 12 weeks postnatal (male preferred). 60 minutes under isoflurane. (90% survival)
2. Recovery for 3-7 days. (90% survival)
3. Multimodal mapping with widefield 1p Ca^{2+} imaging on day 2 or 3. (100% survival)
4. Window implantation surgery. 90 minutes under isoflurane. (75% survival)
5. Recovery for 7-10 days. (90% survival)
6. Habituation to head-fixation and wheel (2-3 days). (100% survival)
7. Spontaneous behavior mesoscope imaging sessions (90 min each). At least three with [5000 x 660 μm] x 7 FOVs at ~3 Hz, and at least three with [660 x 660 μm] x 4 FOVs at ~10 Hz. Interleaved with passive auditory and passive visual stimulation sessions. Up to 3 sessions per day, for a total of 6-9 days of imaging (2-3 weeks). (100% survival)
8. Mice trained in the behavioral tasks (~3-6 weeks). (67% success)
9. Mice imaged on mesoscope while performing multimodal behavior (3-5 sessions over 1-2 weeks). (90% success)
10. Mice imaged on mesoscope, with targeted optogenetic inhibition of cortical subregions, while performing multimodal behavior (3-5 sessions over 1-2 weeks). (90% success)

Total time (t0 = surgery 1): 15 weeks (~3.5 months)

Age range of mouse: 8 to 27 weeks postnatal

Overall survival rate to stage 10: $0.9 * 0.9 * 0.75 * 0.9 = 55\%$

Overall success rate to stage 10: $0.9 * 0.9 * 0.75 * 0.9 * 0.67 * 0.9 * 0.9 = 30\%$

Estimated initial sample size of mice needed to get n=6 for spontaneous and passive: 10

Estimated initial sample size of mice needed to get n=6 for behavior: 20

*Note: Perform additional widefield multimodal mapping sessions and vasculature to Allen Common Coordinate Framework (CCF) coalignment and registration approximately once every 4 weeks or as needed.

Protocol II.

Headpost surgery: “Side mount”

1. Weigh the mouse and prepare 1 mL ringers, 6.0 mg/kg Meloxicam SR, and 0.5 mg/kg Buprenorphine SR.
2. Anesthetize the mouse with 2-4% isoflurane in the induction chamber.
3. Mount mouse in stereotax and rotate 22.5 degrees to the left. Reduce isoflurane to 1.4% at vaporizer, set oxygen flow rate to ~1.5 L per minute.
4. Apply ophthalmic ointment, adjust mouse position on heating blanket, place temperature probe in rectum, and stabilize temperature at 35.5 degrees C. Re-adjust head and neck position until breathing is regular and not jerky.
4. Inject Meloxicam SR and Buprenorphine SR subcutaneously.
5. Shave dorsal and right temporoparietal surface of head and remove residual hair with Nair and surgical spears. Sterilize area three times with alternating cottonswab-applied betadine solution and 70% ethanol wipes. Be careful to clean residual Nair away from eyes with Ringers.
6. Use sharp-tipped surgical scissors to cut skin along the sagittal line from just behind lambda to the middle of the olfactory bulb. Next, cut along the parasagittal arc exposing 1-2 mm of skull over the left cortex. Make a final parasagittal cut deep over the right side of the skull to just below the auditory cortex, then follow closely around the right eye to join the initial cut over the right anterior snout.
7. Clip and retract skin around the opening with 4-6 thin “bulldog” clamps. Use #3 forceps tips, cotton swabs, non-woven sponges, and angled broad edge of #23 scalpel blade to remove periosteum, clean surface of skull, and score expected headpost contact surface.
8. Make a parasagittal cut separating temporal muscle from right parietal ridge, then use blunt back edge of 90 degree angled hook and broad edge of scalpel to tease muscle away from

ridge along its entire length. While holding muscle with #3 forceps, use fine straight spring scissors to cut away the entire muscle from posterior to anterior along the dorsal surface of the zygomatic process, passing closely behind the eye while taking care to avoid rupturing ophthalmic artery. Move 2 bulldog clamps from ventral (right) skin flap to residual edge of muscle so that entire flat lower section of temporal bone is exposed down to corner interface with zygomatic process. If a bleed erupts, place ringers-soaked Vetspon onto soft-tissue pocket and leave for at least 30 seconds, then remove carefully with #3 forceps. Inject subcutaneous Ringers as necessary to supplement for lost blood.

9. Use broad-side of 1.4 mm diameter drill bit to round-off entire length of parietal suture, pick off stray bone shards with #3 forceps, and clean the surface of the skull. If the mouse will be used primarily as widefield preparation, smooth surface additionally with rotary polishing tips.

10. Clean and score contact surface of headpost, then place over surface of skull in intended attachment position. Medial edge of headpost perimeter should be 1-2 mm left of and parallel to midline. Back edge of headpost should be posterior to lambdoid sutures, front edge of headpost should be over olfactory bulbs, eye should be close-in and centered in eye-loop, and ventral (right) edge should be tight and low over and PAST zygomatic process. Carefully note areas where additional skull surface needs to be exposed and cleaned in order to attach headpost. Also note where the zygomatic process crosses the opening of the headpost (important for the upcoming headpost modification step).

11. Use the broad edge of scalpel blade to retract soft tissue and periosteum where needed to make room for headpost attachment, reposition bulldog clamps, and clean and dry skull surface with cotton swabs and non-woven sponges.

12. Place the headpost ventral (right) edge upwards (contact surface facing you) in a small metal crinkle dish. Add UV curable dental cement to the ventral edge, allow gravity to pull in downwards to form a “curtain”, UV cure, and repeat until the edge of dental cement runs along the position of the zygomatic process observed in step 9 (above).

13. Re-check headpost positioning on skull as in step 10 (above), then re-dry skull and headpost contact surfaces. Apply a thin coating of UV curable dental cement (Applicap, 3M) with the spatula along the entire contact surface perimeter of the headpost and place firmly onto

the skull. While applying pressure with one hand, use the other to irradiate dental cement with pulsing ultraviolet (UV) light for ~30 seconds. Forceps can also be used to secure headpost in position after initial contact is formed. Be careful to keep cement away from eyes.

14. Apply remaining dental cement (or deploy 2nd capsule as needed) around the interior perimeter of the headpost, filling all gaps with the skull. Use the spatula to remove excess cement from the skull, then UV cure as in step 13 (above).
15. Use a 1.4 mm diameter drill-bit to remove dental cement along the ventral edge of preparation until a smooth continuous bridge is formed between the zygomatic process, the dental cement, and the headpost.
16. Apply a thin layer of Zap cyanoacrylate (“super-glue”) to the entire surface of the skull and UV cure/let-dry for 15-30 minutes. If the mouse is to be used primarily for wide-field 1p imaging, apply Norland UV curable glue after 1-3 days. In either case, cover Zap with Kwik-SIL (WPI), making sure to include an extended “tab” over the arm of the headpost to enable easy removal.
17. Remove bull-dog clamps, dry skin and push upward around the exterior perimeter of the headpost. Attach skin to the headpost with Vetcbond super-glue. Inject 1 mL ringers subcutaneously, un-rotate and remove mouse from stereotax. Return the mouse to their home cage in a heated incubator unit for at least 24 hours. Inject 1 mL ringers subcutaneously every 24 hours until postoperative weight stabilizes.

Total time: 50-70 minutes

Protocol III.

Cranial window surgery: “Side mount”

1. Surgery to be performed between 3 and 7 days after headpost implantation, after the initial round of widefield multimodal mapping.
2. Inject 10 mg/kg Dexamethasone and 10 mg/kg Baytril subcutaneously on the day before surgery and also on the day of surgery, ~2 hrs prior to placing the mouse on the stereotax.
3. Re-weigh the mouse and prepare 0.5 mg/kg Buprenorphine, 17.5 µL of 25% mannitol heated to 30° C, and 1 mL ringers.
4. Place mouse in induction box under 2-4% isoflurane until fully anesthetized and then transfer to stereotax with head rotated 22.5 degrees to the left so that left arm of headpost is parallel to support arm mounted on breadboard clamped to base of stereotax. Use two 82 degree countersunk 2/56 x 1/4 " screws (McMaster-Carr, MS51959-3D) to affix headpost to support the arm so that the skull does not move during drilling. Make sure that tightening screws does not move headpost relative to skull by iterative micropositioning of head and support arm.
5. Apply ophthalmic ointment and insert a rectal temperature probe. Inject Buprenorphine SR and mannitol subcutaneously. Do NOT inject Meloxicam SR, as N-SAIDs are contraindicated for coadministration with Dexamethasone. Place non-woven sponge under rear paws and remove Kwik-SIL from skull/headpost. Sterilize the skull with betadine and 70% ethanol wipes.
6. Clean 3 custom cranial windows (9 mm radius bend for targeted imaging of ventral-temporal areas and/or imaging of multiple small fields-of-view (FOVs), or 10 mm radius bend for large FOV imaging) by placing them first in a plastic weigh-dish full of 70% ethanol, then rinsing them in ringers and submerging them in ringers in a second plastic weigh-dish.
7. Dry one window by dabbing on sterile surgical drape, then place on skull preparation, centered inside the headpost perimeter. Use a fine point permanent pen to mark the perimeter of the window. Remove and re-clean the window.

8. Prepare 20-30 mL of ice-cold Ringer's solution. Increase oxygen flow-rate to 1.7 L/min and lower isoflurane to 0.3% to maintain regular breathing and decrease intracranial pressure.

9. Use a 0.7 mm diameter drill bit to mark corner positions of the window based on pen markings, then to lightly trace the window perimeter. Gently expand the traced perimeter as much as necessary to fit the entire window without removing too much dental cement, as this can destabilize the headpost.

9. Switch to a 1.4 mm diameter drill bit and remove the next ~80% of skull thickness by tracing slowly and continuously around the perimeter of the craniotomy. Alternate with a 0.9 mm diameter bit as necessary. Continue until vasculature is clearly visible through the skull.

10. Switch to 0.5 mm diameter drill bit and thin skull around perimeter until skull cap is sparsely connected, then switch to manual tools to complete craniotomy. Use 90 and 45 degree micropoints (Fine Science Tools) in a coordinated manner to separate the skull cap around the entire perimeter. Test for separation by pushing down on the skull cap at each location. When bleeds occur, apply large volumes of ice cold ringers across the surface and wick out with non-woven sponge – only use Vetspon when absolutely necessary to stop large bleeds, as formation of clots below the skull cap will significantly impair local window clarity.

11. Prepare one window for placement by carefully drying with the tip of a KimWipe and placing in the correct orientation on the surface of the surgical drape. Attach a left-side stereotaxic microinjection arm (Kopf) and position then rotate-out a 3D printed window stabilizer attached to the end of an injection needle so that it can be quickly repositioned as necessary once the window is in-place. Increase O₂ flow rate from 1.25 to 2.0 L/min, and decrease isoflurane concentration from 1.5 to 1.0% for the remainder of the surgery. This may help to decrease intracranial pressure and to elevate respiration during the final, critical steps of the window implantation.

12. Remove the skull cap by placing the 90 degree micropoint at the rostral limit and the 45 degree micropoint at the caudal limit just right of the sagittal sinus, then lifting the skull up from the caudal limit until the sagittal sinus is pulled away from the dura. Re-lower the skull to remove tension on the vasculature, then re-lift skull slightly higher than the first lift to tease the skull vasculature off the skull so that it remains on the dura. Repeat these steps until the skull is free

of the dura and vasculature. Use the rostral micropoint to prevent the skull from digging into the brain as the caudal edge is raised, then lift together until the skull is cleared.

13. Perfuse surface of brain with ice-cold ringers for ~30 seconds or until all bleeds have stably ceased, using fresh non-woven sponges to flow and wick liquid off opposite side of preparation. Use Vetspon sparingly and only as necessary while continuously perfusing. If a significant blood clot occludes a large area, attempt to remove it with #5 forceps but be extremely careful not to damage dura.

14. Place window on surface of brain with two pairs of #3 forceps. Position 3D printed window stabilizer near center of window at ~22.5 degree angle to the right and adjust until even pressure is achieved across the entire window-brain interface. Iteratively lower and raise stabilizer by small amounts with stereotax until visual clarity and contrast of blood vessels is maximized and breathing stabilizes. Rinse with ice-cold ringers and then thoroughly dry with fine-rolled KimWipe corners and/or sugi spears.

15. Apply Flow-It ALC around the perimeter of the window then UV cure. Make sure that there are no bubbles or gaps. Preemptively apply UV light in areas where there might be a risk of Flow-It invading the preparation under the window.

16. Apply Loctite 4305 around the outer edge of Flow-It, and also at the interface between dental cement and skull, being very careful not to contaminate the surface of the window. UV cure once more.

17. Remove window stabilizer slowly to minimize window rebound. Clean the window with ringers, dry, then apply KwikCast to cover the window, leaving a tab for easy removal over the headpost arm.

18. Subcutaneously inject 1 mL ringers, remove mouse from stereotax, and place in heated recovery unit for at least 24 hours. Subcutaneously inject Meloxicam SR on Day 1 post-surgery, along with additional ringers as needed for post-operative weight stabilization.

Total time: 60-90 minutes

Protocol IV.

Mounting a headpost and window implanted mouse onto Mesoscope or Widefield rigs

1. If mounting a dorsal mount preparation mouse, attach two horizontally aligned or “orthogonal” support arms to the breadtable on either side of the running wheel (see Figs. 1a, S1c top).
2. If mounting a side mount preparation mouse, attach one 22.5 degree-angled headpost attachment on the left side of the running wheel (see Figs. 1b, 1c bottom).
3. Insert and fully tighten, and then remove countersunk test screws into the support arm to test the threading. If mounting a dorsal mount preparation mouse, affix a “test” headpost to both support arms to ensure that their 3D alignment is correct, before attempting with the mouse.
4. Place the mouse centered on the running wheel and hold the base of its tail with your right hand.
5. If mounting a dorsal mount preparation mouse, use your left hand to place the left wing of the headpost into the support arm screw slot (see Fig. S1b, c top, e). Hold the wing in the slot with your thumb, and use your right hand to affix the right wing to its support arm with a single screw.
6. Use a second screw to affix the left wing, then tighten both sides fully in step.
7. If mounting a side mount preparation mouse, use your left hand to place the left wing of the headpost in the support arm slot. Then, use your left thumb to hold the tip of the wing in the slot while using your right hand to insert and tighten a screw in the hole proximal to the mouse’s head. Then, use your right hand to hold the base of the mouse’s tail and your left hand to insert and tighten a screw in the hole distal to the mouse’s head.
8. Fully tighten both screws in step.
9. If mounting on the mesoscope, place a flat 3D printed plate fitted to the headpost that you are using onto the preparation, and hold in place bilaterally with 3-pronged lab clamps attached to support arms with quick-ties.. With UV and IR illumination on, use LabView NI MAX software to

align cameras and ensure that the mouse's face and eyes/pupils are fully visible on both the left and right sides.

10. Mix 1:1 black and white 170 FAST CURE Sylgard in a plastic weigh boat. While holding the flood light fiber in your left hand, use the wooden back-end of a cotton swab to carefully drip Sylgard into the interface between the 3D printed plastic (black PLA) plate and the titanium headpost. Make sure that there are no gaps, and that you do not contaminate the cranial window. Wait 5-6 minutes for curing to complete, add water to full meniscus height to test for leaks before proceeding with imaging.

11. If mounting on the widefield, attach the 3D printed light blocking cone (left half for multi-modal, full circumference for behavioral experiment) by pressing it onto the edge of the headpost and using an inverted 3-pronged lab clamp attached to the imaging lens with velcro to grip and secure the top edge of the cone before proceeding with imaging.

Protocol V.

Allen CCF, widefield, vasculature, and 2-photon co-alignment and registration (see Figs. 2, 3, S2; Suppl Movies S2, S3)

1. Acquire co-aligned blood vessel, skull, and multimodal widefield reference images (see Multimodal Mapping supplementary protocol).
2. Create blood vessel and skull images by loading 30 s (1500 frames at 50 Hz) widefield movies into Fiji, auto-adjusting brightness and contrast, and creating standard deviation z-stack projections.
3. Create stimulus triggered mean dF/F (here F_0 is equal to the global 10th percentile of fluorescence intensity) images for each 5 min (15000 frames at 50 Hz) sensory stimulation movie by averaging the 1 s baseline-subtracted Ca²⁺ fluorescence response in a 1 s window following each stimulus onset using custom Matlab code ("SensoryMapping_Vickers_Jun2520.m). Adjust stimulus timing signal detection threshold for each modality as needed.
4. For each multimodal mapping session, load skull, blood vessel, and multimodal dF/F images into Fiji and make sure that image size and resolution are the same for all images before proceeding.
5. Create a master overlay image by starting with the blood vessel image. Then proceed through each overlay image with the following substeps: i) Threshold image so that the saturated area is contiguous in the target area and has an outline that matches its shape in the Allen CCF. ii) Create a mask. iii) Create selection. iv) Use the magic wand tool to select the region of interest. v) Select master blood vessel image. vi) Press shift+e to place selection outline as overlay. vii) Select Image/overlay/flatten and save new image.
6. It may be necessary to perform step 5 (above) for the skull image by manually selecting bregma and lambda with Fiji circle drawing tool.

7. Open custom Matlab code for CCF alignment and check to confirm that it's "on path" (align_recording_to_allen_Vickers_affine_Jan0120.m, or align_recording_to_allen_Vickers_pwl.m; adapted code from Shreya Saxena and Matt Kaufman, 2018).
8. Navigate to the folder containing outputs of steps 5 and 6.
9. If aligning a "Crystal Skull" preparation or an "A1/V1/M2" preparation with fewer than 6 alignment points, use code for "affine" transformation. If aligning an "A1/V1/M2" preparation with more than or equal to 6 alignment points, use code for "pwl" (piecewise linear) transformation.
10. Run "computeAllenDorsalMap.m" to create "allenDorsalMap.mat", and create a string array called "alignareas" containing a list of CCF areas whose centers you will designate based on your master overlay image. Each entry will be in the form "Cortical_hemisphere Area_name"; for example: "R VISp1", "R AUDp1", or "R SSp-bfd1". Copy these files into your working directory.
11. Load your image by double-clicking its name in the "Current folder" window.
12. Convert your image to grayscale and auto-adjust brightness and contrast by typing "im0=mat2gray(imageName)", followed by im0=imadjust(im0).
13. Load "alignareas", "areanames", and "dorsalMapScaled" by double-clicking on "preprocessed_allenDorsalMap.mat" and "alignareas" in the "Current Folder" window.
12. Run the "align_recording.....m" code by typing "tform = align_recording_to_allen_Vickers_affine(im0,alignareas,true)
13. Select points on the master image as requested.
14. Program will generate output images with CCF overlaid on the master image with reference points shown as red "x", user-selected points as blue "o". For affine transformation, use "...inverse.png" output so see CCF overlay on original coordinate system.

15. Run custom code to warp and align mesoscope meanImage with cellMap onto master overlay image with CCF based on user-identified common vasculature intersections.
16. For each Suite2p-identified cell in the cellMap, assign a coded CCF area name (i.e. a dictionary with a unique number and color identifier for each area).

Supplementary Methods and Materials

Overall workflow, Protocol I

Approximate Costs—Single Behavior Rig

Product Description	Manufacturer	Quantity	Unit Price	Extended Price
25 Watt power supply	Tucker Davis	1	325	325.00
Electrostatic loudspeakers	Tucker Davis	4	195	780.00
Electrostatic loudspeaker drivers	Tucker Davis	2	650	1,300.00
PCI 4461 Sound card	National Instruments	1	4,805.00	4,805.00
SCB 68 with Connector Cable	National Instruments	1	478.00	478.00
PCIe 6321 Multifunction I/O	National Instruments	1	690.00	690.00
810 nm narrow bandpass filter	Midwestern Optical Systems	1	141	141
Genie Nano M2050 Mono	Teledyne Dalsa	1	878.43	878.43
NE-500 Programmable OEM Syringe Pump	New Era Pumps	7	495	3,465.00
US OEM Starter kit	New Era Pumps	7	25	175
C-Mount 55mm Telecentric Fixed Focus Lens COTEC55	computar	4	327.52	1,310.08
Assorted Optomechanical Posts and breadboard (approximate)	Thorlabs	varied	N/A	488.88
LCD Monitor with Blanking Apparatus (Includes Arduino Micro-Controller) (approx.)	Amazon/Arduino	varied	N/A	120.00
Rotary Encoder	KAMAN AUTOMATION INC	1	217	217.00
Custom Cylindrical Treadmill	Public Missiles LTD	1	84	84.00
Lick Detection Unit	Custom Build	1	1000	1000.00
Computer for Behavior (Intel i5 or better, 32 GB RAM or better) (approx.)	User Preference	2	700	1400.00
Assorted Electrical Components, Misc. Hardware, Adapters, etc.	Varied	varied	N/A	700.00
Power 1401	CED	1	5697.60	5697.60
			Total	24,054.99

Design software

autodesk.com: Inventor and Fusion
emachineshop.com

3D printed titanium headposts, shot-peening finish

i.materialise.com/
sculpteo.com/
~\$35-60 per headpost

3D printed light deflectors and accessories

makergear.com
black PLA, 0.35 mm nozzle

Kopf stereotax

926-B mouse nose/tooth bar assembly
907 mouse anesthesia mask

Widefield imaging

Redshirt Imaging, DaVinci SciMeasure & Turbo-SM64
PCO.edge 5.5 M-AIR-CLHS-PCO, & CamWare 4.0
Tamron SP 90mm f/2.8 Di Macro 1:1 VC USD Lens for Nikon F

Headpost surgery, Protocol II

Mouse electric trimmer combo kit with detailer (CL9990-1201, Kent Scientific)
Nair sensitive hair removal cream (amazon.com)
PurSwab 3" small pointed ESD foam swabs (amazon.com)
Vetoquinol Nutri Cal (4.25 oz Paste; amazon.com)
Puralube Vet Ophthalmic Ointment (amazon.com)
3M Vet Bond (amazon.com)
Scalpel blades - #23 (10023-00; Fine Science Tools)
Kwik-Cast sealant (World Precision Instruments)
Kwik-SIL sealant (World Precision Instruments)
Pacer Technology (Zap) Slo-Zap (Thick) Adhesives, 2 oz (amazon.com)
Micro-bulldog clamp for mice (INS600119-2, Kent Scientific)
RelyXUniCem Aplicap Refill A1 20/Bx 3M ESPE Products (036090-3789981; Henry Schein DBA Butler Animal Health)
Disposable aluminum crinkle dishes with tabs, 8 mL (12577-081; VWR)
3M Aplicap Applier Activator/Applier Set, 37160
HP 1RF-009 Round Stainless Steel Burs Pk/10 (1RF-009-HP (All4Dentist)
Small homeothermic blanket system with control unit (Q-21090, Harvard Apparatus)
Gelfoam for cessation of bleeding (NC1061303, Fisher Scientific)
Applicator cotton tipped his non-sterile, 3 inch, wood handle (Henry Schein)
Dumont #3 forceps (11293-00; Fine Science Tools)
Angled 80 deg long probe (10140-03; Fine Science Tools)
Fine scissors, tungsten carbide, straight (14568-09; Fine Science Tools)
Vannas spring scissors – 2.5 mm (15000-08; Fine Science Tools)

Cranial window surgery, Protocol III

Labmaker.org, "Crystal Skull – One Million Neurons" (Tony Kim, Yanping Zhang and Mark Schnitzer; https://www.labmaker.org/products/crystal-skull?_pos=1&_sid=84c16cded&_ss=r)

TLC International custom cutting, Phoenix-600, 0.21 mm Schott D263T Glass (9849 North 21 Avenue, Phoenix, AZ; A1/V1/M2)

GlasWerk Inc, custom 9-12 mm radius glass bending (29710 Avenida de las banderas, Rancho Santa Margarita, CA; A1/V1/M2)

Loctite 4305 LT cure ad 1oz bottle (LT303389, Krayden)

Dynarex non-woven sponge, N/S 4Ply (amazon.com)

Osada EXL-M40 brushless micromotor system (EXL-M40; Dorado dental supply)

Flow It ALC Flowable Syringe WO Value Pack 6/Pk (726240, Henry Schein)

Micro-point, angled 90 degree long (10065-15; Fine Science Tools)

Micro-point, angled 45 degree long (10066-15; Fine Science Tools)

Rig mounting, Protocol IV

Mil Spec St Steel Phillips Flat Head Screws, 82 degree (96877A18, McMaster-CARR)

Dow Corning Sylgard 170 Fast Cure Silicone Encapsulant Black 210 mL (Ellsworth Adhesives)

Posts (Thorlabs; TR6-P5)

Ball joint (Thorlabs; SL20)

Post holder (Thorlabs; PH2E)

Clamp fork (Thorlabs; CF125)

Platform (Thorlabs, MB4)

Motorized linear stage, 25 mm range, 104 mm/s, 48V (X-LSM025B-E03-KX14A; zaber.com)

Multimodal mapping

Eyoyo 15.6" inch Gaming Monitor 1920x1080 HDR Display Second (newegg.com)

E-650 Piezo Amplifier for Multilayer bending actuators, 18 W

PICMA® multilayer piezo bending actuator, 2000 µm travel range, 45 mm × 11.00 mm × 0.55 mm, stranded wires (PL140.11; physik instrumente.store)

Tucker Davis ES1 Free Field Electrostatic Speaker

Tucker Davis ED1 Electrostatic Speaker Driver

Oben BD-0 mini ball head (amazon.com)

Locking ball and socket mount, ¼"-20 threaded (TRB2; Thorlabs)

Mounted LED 470 nm (760 mW, 1000 mA) (M470L4, Thorlabs)

Dichroic (T495lpxr, chroma.com)

Excitation filter (ET470/40x; chroma.com)

Emission filter (ET525/50m; chroma.com)

Kinematic fluorescence filter cube (DFM1, Thorlabs)

ScanImage 2p acquisition

Spectra Physics Mai Tai HP-244 tunable femtosecond laser

<https://www.spectra-physics.com/en/f/mai-tai-ultrafast-laser>

Thorlabs Mesoscope

https://www.thorlabs.com/newgroupage9.cfm?objectgroup_id=10646

ScanImage 2018-2021 (Vidrio Technologies)

<https://vidriotechnologies.com/scanimage/>

<http://scanimage.vidriotechnologies.com/display/SIH/ScanImage+Home>

Computing resources

EVGA GeForce RTX 3080 Ti FTW3 Ultra Gaming, 12G-P5-3967-KR, 12GB GDDR6X, iCX3 Technology, ARGB LED, Metal Backplate (B0922N253, amazon.com)

Tesla V100 PCIe 32GB GPUs