Supplementary Materials

The COMBO window: A chronic cranial implant for multiscale circuit interrogation in mice

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Files:

Supplementary File 1: COMBO_cup.stl
Supplementary File 2: COMBO_flat.stl
Supplementary File 3: COMBO_lateral.stl
Supplementary File 4: COMBO_posterior.stl
Supplementary File 5: COMBO_anterior.stl
Supplementary File 6: COMBO_Q1.stl
Supplementary File 7: COMBO_Q2.stl
Supplementary File 8: COMBO_Q3.stl
Supplementary File 9: COMBO_Q4.stl
Supplementary File 10: Head_plate.dwg
Supplementary File 11: Head_plate_holder.sldprt
Supplementary File 12: Head_plate_holder_top.dwg
Supplementary File 13: Head_plate_holder_bottom.dwg
Supplementary File 14: Brain_mold.stl

Videos:

Supplementary Video 1: Facial videography during a trial of sucrose delivery
Supplementary Video 2: Facial videography during a trial of quinine delivery
Supplementary Video 3: Brain-wide fUS activity in awake mice in response to visual stimulation
Supplementary Video 4: Facial videography and two-photon imaging in the retrosplenial cortex during locomotion

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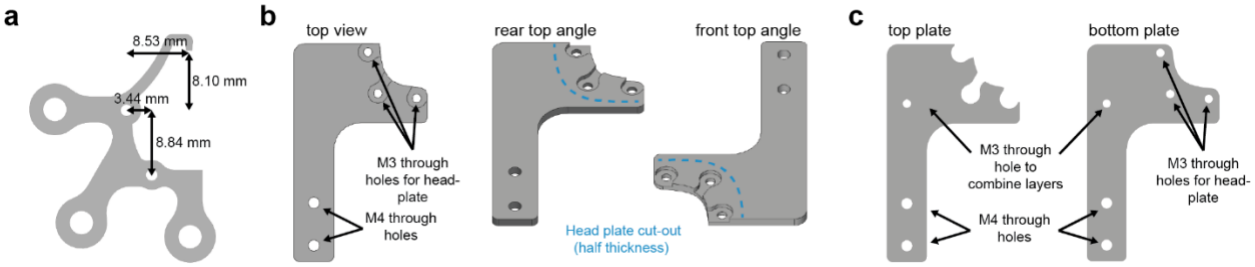
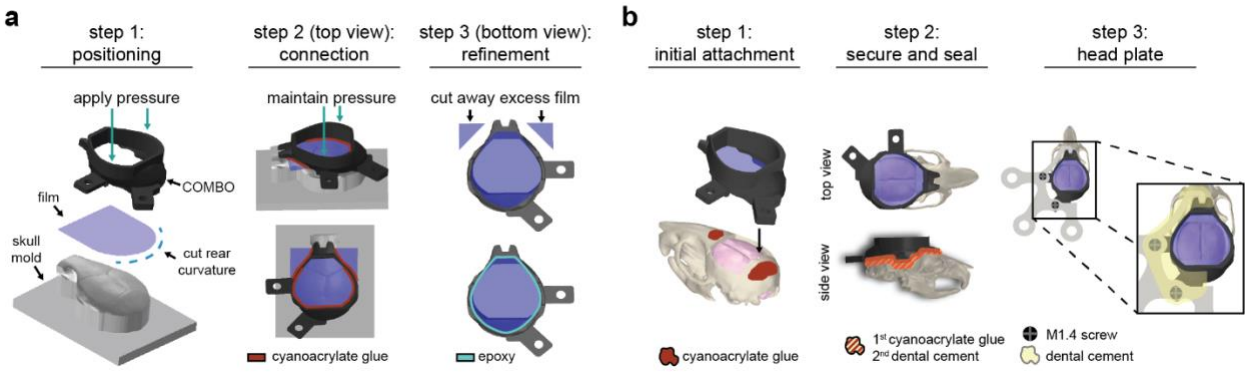
Appendix 1: COMBO window preparation and installation protocol



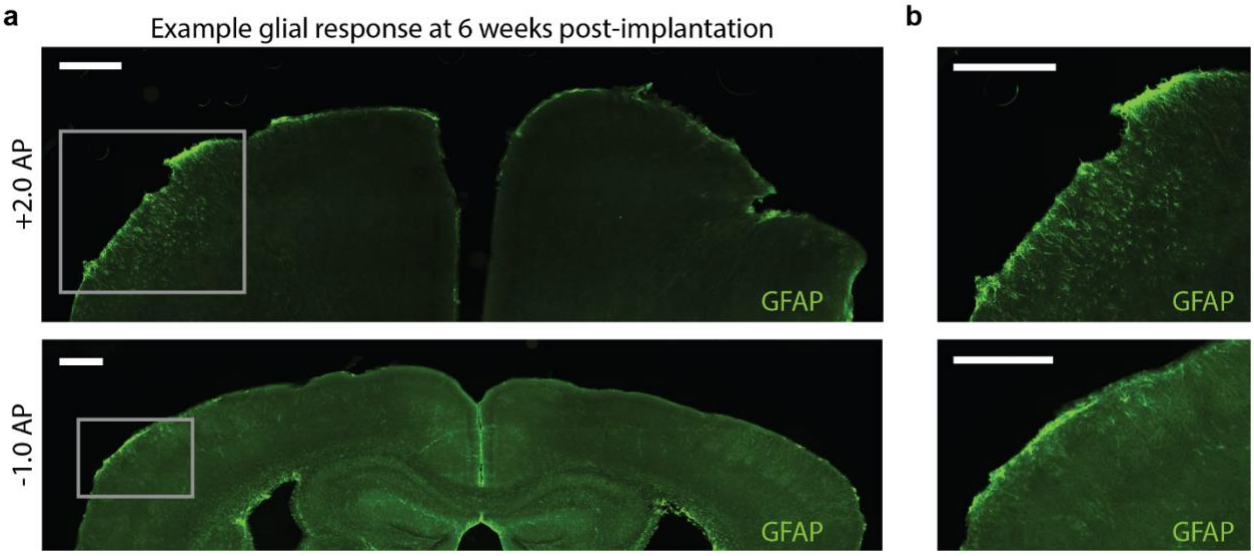
Supplementary Figure 1: Head fixation part details

a Computer-aided design of the standard COMBO window head-plate (Supplementary File 10). The head-plate attaches to the implant via two M1.4 through holes at the side and rear, as well as a peg in the front. Other custom head plate designs with the same features and relative distances can also be used for head fixation. **b** The standard head-plate holder design (Supplementary File 11) consists of a single metal plate with the head plate outline cut halfway through the total thickness. Threaded M3 screws are welded into the head-plate holes and grinded flush with the underside of the plate. M4 through holes allow for attachment to other commercial or custom parts for further stabilization. It is recommended that a machine shop helps with the fabrication of this part. **c** An alternative head-plate holder design consists of a top and bottom plate (Supplementary Files 12-13) that can each be laser cut and joined together with no custom fabrication. M3 screws can be used to secure the two layers together, and M4 screws to attach the head-plate holder to other commercial or custom parts. Additional M3 screws can be attached via the underside of the holder using glue/epoxy.



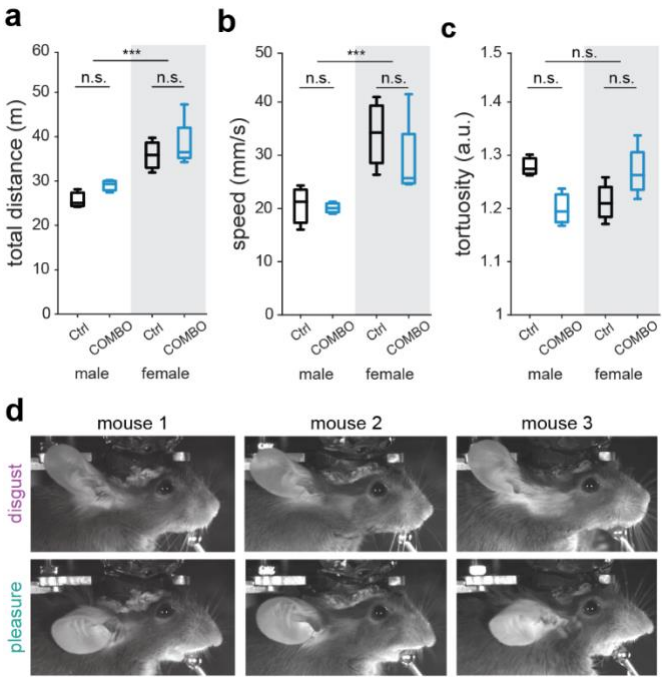
Supplementary Figure 2: COMBO window assembly and installation instructions

a Three-step diagram of the preparation of the COMBO window. Using the skull mold (Supplementary File 14) is recommended but not required for proper assembly. **b** Three-step diagram of the installation of the COMBO window after a cranial window has been created. The head plate can be installed at the same time as Steps 1-2 or at a later date. Detailed methods for both of the procedures are provided in Appendix 1.



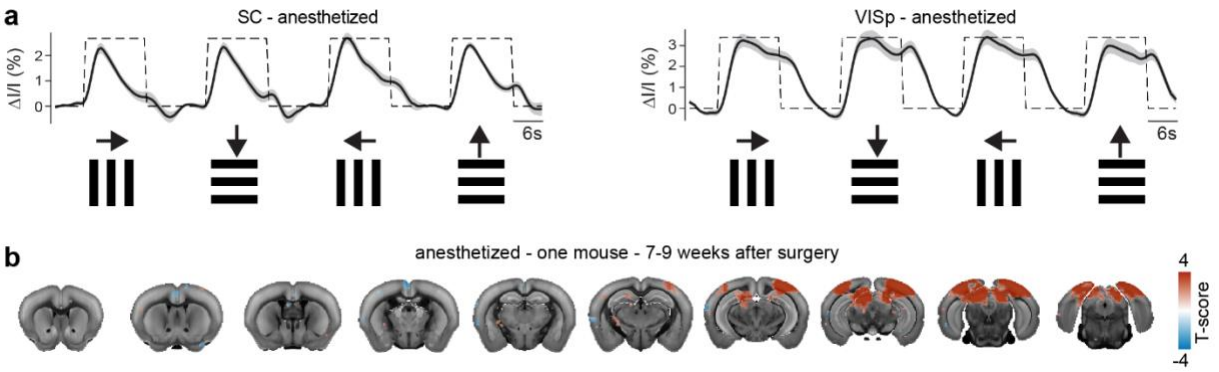
Supplementary Figure 3: Sporadic GFAP fluorescence was observed in mice implanted with the COMBO window

a Glial fibrillary acidic protein (GFAP) fluorescence in two example slices (top: bregma +2.0 mm AP, bottom: bregma -1.0 mm AP) of mice at 6 weeks after being implanted with the COMBO window. In both images, a localized increase of GFAP fluorescence can be seen in the left hemisphere. **b** Zoomed-in images of the elevated GFAP signal indicate that the immune response was found mostly in fibers located at or near the pial surface. Scale bars represent 500 µm.



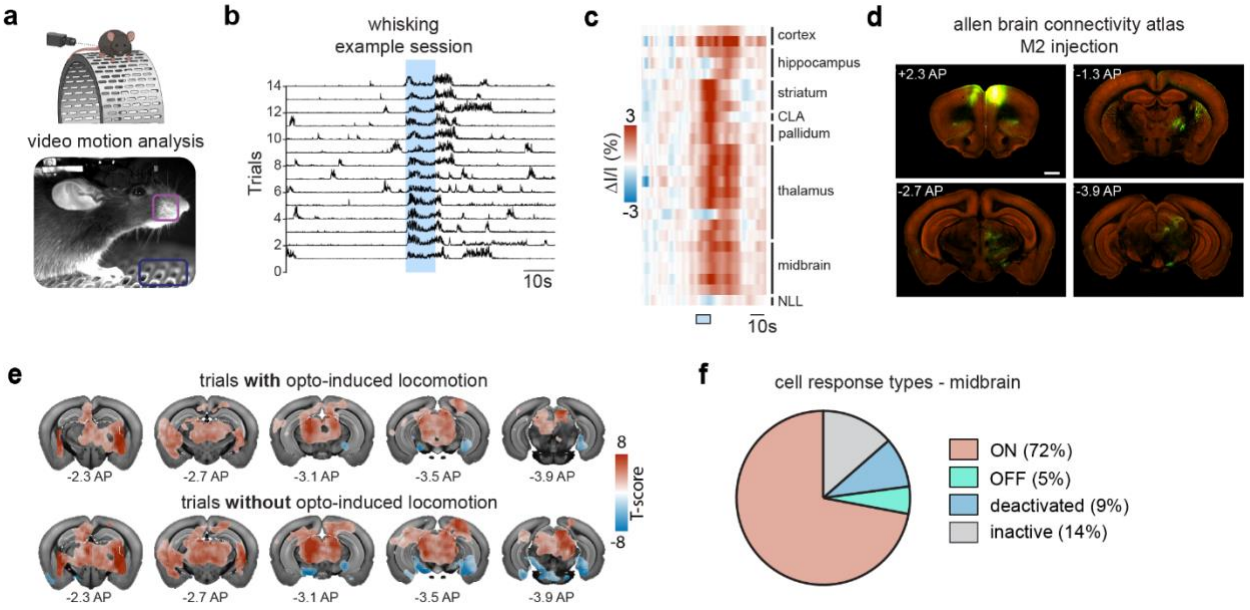
Supplementary Figure 4: Behavioral effects are consistent across sex and individuals

a-c The total distance (**a**), speed (**b**), and tortuosity (**c**) of control and COMBO window mice separated into male (n = 3) and females (n = 4). Boxplots represent the median (center line), 25th and 75th percentiles (lower and upper box), and the 1st and 99th percentile (whiskers). Two-way ANOVA on ranks with main effects of sex and cranial window. Main effect of sex: *** $p < 0.001$. Post hoc pairwise t-tests, Bonferroni corrected: n.s. $p > 0.05$. **d** Example prototypical disgust and pleasure facial expressions exhibited by three animals with a “cup” version of the COMBO window installed. Key features of the elicited disgust face include a flaring back of ear and an upturned snout, and of the elicited pleasure face include the forward movement of the ear and a downturned snout.



Supplementary Figure 5: The COMBO window enables chronic brain-wide acquisition of fUS data

a fUS signal in the superior colliculi (SC) and primary visual cortex (VIS) of anesthetized mice covaries in response to drifting gratings in all four cardinal directions (N = 5 mice, n = 32 sessions). The dark black line and light gray shaded area represent the mean ± s.e.m. across sessions. **b** GLM results from 8 sessions of a single mouse recorded 7-9 weeks after surgery overlayed on the Allen Brain Atlas. Only voxels with an average T-score > 2 are displayed.



Supplementary Figure 6: The COMBO window facilitates longitudinal experiments to interrogate neural circuits underlying behavior

a Facial videography was used to monitor animal behavior on a running wheel. Regions-of-interest (ROIs) placed over the whisker pad (violet) and wheel (green) were utilized to capture whisking activity and locomotion, respectively, induced by optogenetic stimulation of the secondary motor cortex (M2). **b** Consecutive trials from the same example session as in Figure 5c showing a robust and reliable increase in whisking in response to optogenetic activation of M2. Each trial was z-scored to a pre-stimulus baseline and rescaled between 0 and 1 for visualization purposes. **c** Region-wise segmented results of the optogenetically-induced fUS activity. Regions are sorted by brain area, and only significantly modulated (correlation between stimulus timing and fUS signal) regions are included (significantly different from zero across sessions, $p < 0.001$, FDR-corrected). **d** Example coronal slices from the Allen Brain Connectivity Atlas (connectivity.brain-map.org/projection/experiment/287995889). AAV tracings after injection into the M2 (1) show widespread axonal projections from M2 to the striatum, the thalamus and the midbrain (2 - 4). Scale bar represents 1 mm. **e** GLM analysis of fUS data in response to optogenetic stimulation of M2 (N = 2 mice, n = 12 sessions). In contrast to Figure 5e, here the trials were separated according to strong or weak locomotor response (see methods for threshold definition) to optogenetic stimulation. Only the voxels with T-scores significantly different from zero across sessions ($p < 0.05$, FDR-corrected) are shown. **f** Pie chart showing the proportion of different cell response types observed in the midbrain (see methods for cell response type definition).

Supplementary Table 1: Open field foraging task statistical analysis

Parameter	Statistical Test	Comparison	Post Hoc Test	P-value
Total Distance	Two-way ANOVA on ranks CW vs Ctrl F(1,13) = 1.93, p = 0.19 Male vs Female F(1,13) = 34.05, ***p = 0.002	COMBO vs Ctrl (Male only)	Bonferroni	p = 1.00
		COMBO vs Ctrl (Female only)	Bonferroni	p = 1.00
Speed	Two-way ANOVA on ranks CW vs Ctrl F(1,13) = 0.75, p = 0.41 Male vs Female F(1,13) = 31.21, ***p = 0.002	COMBO vs Ctrl (Male only)	Bonferroni	p = 1.00
		COMBO vs Ctrl (Female only)	Bonferroni	p = 1.00
Tortuosity	Two-way ANOVA on ranks CW vs Ctrl F(1,13) = 1.27, p = 0.29 Male vs Female F(1,13) = 0.03, p = 0.86	COMBO vs Ctrl (Male only)	Bonferroni	p = 0.06
		COMBO vs Ctrl (Female only)	Bonferroni	p = 0.48

Supplementary Table 2: Facial expression statistical analysis

Parameter	Statistical Test	Comparison	P-value
Disgust	Wilcoxon rank sum test	Quinine vs Neutral	p = 0.0001
		Sucrose vs Neutral	p = 1.00
Pleasure	Wilcoxon rank sum test	Quinine vs Neutral	p = 0.92
		Sucrose vs Neutral	p = 0.0011

Supplementary Table 3: Author Contributions

	BJE	DS	PW	BJ	BS	AR	NG	TF	EM
Conceptualization	X	X							X
Data Curation	X	X	X						
Formal Analysis	X	X							
Funding Acquisition	X	X					X	X	X
Investigation	X	X	X	X	X	X			
Methodology	X	X	X	X			X	X	X
Project Administration	X								X
Resources							X	X	X
Software	X	X							X
Supervision	X						X	X	X
Validation	X	X	X						X
Visualization	X	X							X
Writing - Original Draft	X	X							X
Writing - Review & Editing	X	X	X					X	X

Appendix 1: COMBO window preparation and installation protocol

1. COMBO Window Preparation

1.1. Download and print one of the COMBO window files (**Supplementary Files S1-9**) and the Brain_mold.stl (**Supplementary File 14)** using a 3D printer.

NOTE: If the 3D printer utilizes supports when printing, they should be placed on the top of the implant frame and on the bottom of the skull mold to ensure smooth contact surfaces between these two parts. The supports should be cut away and the contact points sanded smooth.

1.2. Thread the two holes of the implant frame using a M1.4 tap. Due to animal safety and physical difficulty, these holes should not be threaded after implantation.

1.3. Cut a square of film (0.125 mm thickness) slightly bigger than the implant frame. Cut a round edge on one side of the film to fit the curvature at the posterior end of the implant frame (**Supplementary Figure 2a, step 1**).

1.4. Place the film on the bottom mold (round part at the back) with the implant frame on top. Apply pressure at the edges of the implant frame downward onto the mold. Try to minimize buckling and ensure that there are no gaps between the film and the implant frame (**Supplementary Figure 2a, step 1**). Multiple attempts to achieve proper positioning may be needed.

1.5. While maintaining pressure, apply a conservative amount of tissue adhesive (low-viscosity cyanoacrylate glue) to the interior rim of the implant frame to attach the film. It is also recommended to apply super glue (high-viscosity cyanoacrylate glue) to the exterior of the frame where any excess film protrudes (**Supplementary Figure 2a, step 2**). After a few minutes (to allow the glue to dry), if these steps have been performed correctly, the implant frame and the film can be removed from the mold together. Other types of glue can also be placed on various interior/exterior edges of the frame according to what is easiest for the user.

NOTE: Avoid covering the film with glue as this can affect the transparency of the window.

1.6. Apply a layer of epoxy to the border of the interface between the film and the implant frame (**Supplementary Figure 2a, step 3**).

1.7. Let the epoxy dry overnight and remove the excess film using a scalpel on the following day. Then, file the hardened epoxy as close to the implant frame as possible (**Supplementary Figure 2a, step 3**). Repeat steps 1.6 – 1.7 until the film is fully secured to the implant frame with no gaps, and is flush with the surface.

NOTE: Before implant installation, ensure that the implant is thoroughly cleaned using 100% ethanol.

1.8. Download the Head_plate.dwg file (**Supplementary File 10**) and laser cut this shape from 1.5 mm stainless steel. A brushed finish will increase the grip of the dental cement.

2. COMBO Window Installation

2.1. Cranial Window Surgery

2.1.1 Prepare the animal for surgery according to locally approved animal licenses and secure the animal's head using a bite bar or stereotaxic frame.

2.1.2. Using sterile scissors cut a 2 cm midline incision through the scalp and periosteum to expose the skull. This incision should reach from just behind the ears to the middle of the eyes.

2.1.3. Detach the periosteum using a cotton-tipped applicator. Then, detach the temporalis and trapezius muscles on the sides and rear of the skull, respectively, using scissors or forceps. Try to maximize the surface area of exposed skull to allow firm attachment of the implant in later steps. Ensure that the skull surface is dry using cotton tipped applicators (use 0.3% H2O2 if necessary).

NOTE: Avoid damaging vessels behind the eyes and at the rear of the skull when detaching the muscles. Damaging such vessels can cause large bleeds and will reduce the animal's chance of survival.

2.1.4. Push the muscles down and secure them to the skull in place using tissue adhesive. Make sure that no gaps remain and that, as mentioned above, as much of the skull stays exposed as possible. Clean debris from the skull surface with a wet cotton-tipped applicator and dry with compressed air.

2.1.5. Set hand drill to ~5000 rotations per minute and mark an outline of the craniotomy on the skull by drilling superficially. Briefly, the extent of the craniotomy can extend the full width of the skull and from the rostral rhinal vein (anterior) to the transverse sinus (posterior) (or even further to the end of the cerebellum). Additional details regarding steps 2.1.5. – 2.1.7. can be found in Brunner et al. 2021 and Hattori et al. 2022.

2.1.6. Continue to deepen the outline by repeatedly moving the drill over the initial groove until the bone island is "floating" on top of the brain. Use compressed air to gently blow away bone debris as needed. Occasionally apply cool saline to the skull to prevent overheating.

2.1.7. To remove the skull, use forceps (e.g. 90 degree) to lift the anterior edge of the bone towards the posterior end. For this, cover the skull with buffer or saline and gently lift the bone island little by little until the soft tissue connections have fully detached from the dura and vessels. The dura should be left intact.

NOTE: There are strong soft tissue connections between the skull and dura along the superior sagittal sinus. Removing the skull too quickly and under too dry of conditions can rip the SSS and cause a potentially fatal bleed. This step can take up to 15 minutes or more for correct detachment without major bleeding.

2.1.8. After removal of the bone, clean the edges of the craniotomy and ensure that the residual bone surface is dry before proceeding with the next steps. Keep the dura moist at all times.

2.2 Implant Attachment and Sealing

2.2.1. Rinse the ethanol-cleaned implant with saline and let it dry on a paper towel.

2.2.2. Place a small amount of super glue on the skull, anterior and posterior to the cranial window. Gently place the implant on top of the cranial window, ensuring that there is secure contact with the areas covered with super glue (**Supplementary Figure 2b, step 1**). The implant should hug the sides and the rear of the skull in this step.

NOTE: Some of the film may make direct contact with the dura during this step, but full contact is not necessary at this point. If sealed properly, the window will fill up with cerebrospinal fluid within a few days (**Figure 1b**).

2.2.3. Once the implant feels secure, place additional super glue in the gaps between the implant and the skull to fully secure and seal the implant to the skull (**Supplementary Figure 2b, step 2**).

NOTE: There is generally less skull exposed at the anterior parts of the head, and it can therefore be difficult to properly seal this section (especially around the eyes). If these sections are not properly sealed, dental cement can easily fall through this gap and onto the brain surface in step 2.2.4.

2.2.4. Prepare dental cement according to the manufacturer's instructions and apply generously around the circumference of the implant and skull (on top of the glue) (**Supplementary Figure 2b, step 2**). Ensure that there are no gaps between the implant and skull. Contact with the sutured skin will largely prevent the animal from opening wounds.

2.2.5. Attach the head-plate to the implant with two M1.4 screws (**Supplementary Figure 2b, step 3**). Prepare additional dental cement and apply to the two screws and front peg to ensure that the head-plate is locked in place on the implant. Additional cement can be placed in the gap between the head-plate and implant for a firmer connection (**Supplementary Figure 2b, step 3**). This step can also be performed in a second surgery after recovery from the craniotomy and implant installation.

2.2.6. Apply silicone (e.g. Kwik-Cast silicone sealant) on top of the film to protect it until imaging.

2.2.7. Reverse the anesthesia and provide post-operative care according to locally approved animal licenses for the required number of days. When the animal is returned to its home cage, it is strongly recommended to remove any overhead gratings in the home cage to avoid interference with the implant/head-plate, and place additional feed on the floor of the cage.

3. Head Fixation and Imaging

3.1. We propose two assembly methods of the head fixation system for securing the mouse to the experimental setup. For the first (preferred) design, download the Head_fixation.sldprt file (**Supplementary File S11**) and manufacture the shape from 2 mm stainless steel from an external provider or workshop. Alternatively, the Head_plate_holder_top.dwg and Head_plate_holder_bottom.dwg files can be used together to construct the same design (**Supplementary Files S12-13**). This approach has the advantage of being compatible with laser cutting. Each part should be laser cut from 1 mm stainless steel and secured together via the rear mounting holes (M4 through holes). In either case, thread the fixation holes (M3 through holes) with an M3 tap and feed M3 screws (with low profile heads) upward through each. Alternatively, the M3 screws can be permanently secured with epoxy or M3 studs can be welded in place. The head fixation should be secured in the behavioral setup prior to the start of habituation.

3.2. Animals should be handled for three days for 10 - 15 min per day prior to being trained on a particular behavioral setup. On the first day of habituation to the setup, allow the mouse to first explore as performed previously. After 5 - 10 minutes, position the animal into alignment with the head fixation screws. Gently lift the head-plate with a pair of forceps onto the pins while supporting the body of the animal with the other hand. Tighten M3 nuts on top of the pins to secure the head-plate, first by hand and then using a socket driver. The duration of a habituation session should increase each day for at least five days.

3.3. On days in which imaging takes place, remove the silicone window protection, and wash the surface of the film with saline and dry with a cotton-tipped applicator. After imaging is complete, it is recommended that the silicone window protection be replaced (while the animal is still head-fixed) until the next imaging session.