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Hybrid Microdrive System with Recoverable Opto-Silicon Probe and Tetrode for Dual-Site High Density Recording in Freely-Moving Mice

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Response letter to the reviewer

Dear Dr. Phillip Steindelr,

We thank you for giving the opportunity to resubmit our manuscript, JoVE60028 "Hybrid Microdrive System with Recoverable Opto-Silicon Probe and Tetrode for Dual-Site High Density Recording in Freely-Moving Mice,".

We confirm that this manuscript is in accordance with the authorship statement of ethical standards for manuscripts submitted to *Journal of Visualized Experiments* (*JoVE*).

Please address all correspondence concerning this manuscript to me at Jun.Yamamoto@UTSouthwestern.edu. Thank you for considering our paper for publication.

Sincerely,

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TITLE:

Hybrid Microdrive System with Recoverable Opto-Silicon Probe and Tetrode for Dual-Site High Density Recording in Freely Moving Mice

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KEYWORDS:

in vivo electrophysiology, chronic recording, hybrid microdrive array, tetrode, recoverable opto-silicon probe, optogenetics, multisite recording, freely moving mice, single unit, local field potential, dorsal hippocampus CA1, dorsal medial entorhinal cortex

SUMMARY:

This protocol describes the construction of a hybrid microdrive array that allows implantation of nine independently adjustable tetrodes and one adjustable opto-silicon probe in two brain regions in freely moving mice. Also demonstrated is a method for safely recovering and reusing the opto-silicon probe for multiple purposes.

ABSTRACT:

Multi-regional neural recordings can provide crucial information to understanding fine-timescale interactions between multiple brain regions. However, conventional microdrive designs often only allow use of one type of electrode to record from single or multiple regions, limiting the yield of single-unit or depth profile recordings. It also often limits the ability to combine electrode recordings with optogenetic tools to target pathway and/or cell type specific activity. Presented here is a hybrid microdrive array for freely moving mice to optimize yield and a description of its fabrication and reuse of the microdrive array. The current design employs nine tetrodes and one opto-silicon probe implanted in two different brain areas simultaneously in freely moving mice. The tetrodes and the opto-silicon probe are independently adjustable along the dorsoventral axis in the brain to maximize the yield of unit and oscillatory activities. This microdrive array also incorporates a set-up for light, mediating optogenetic manipulation to investigate the regional- or cell type-specific responses and functions of long-range neural circuits. In addition, the opto-silicon probe can be safely

recovered and reused after each experiment. Because the microdrive array consists of 3D-printed parts, the design of microdrives can be easily modified to accommodate various settings. First described is the design of the microdrive array and how to attach the optical fiber to a silicon probe for optogenetics experiments, followed by fabrication of the tetrode bundle and implantation of the array into a mouse brain. The recording of local field potentials and unit spiking combined with optogenetic stimulation also demonstrate feasibility of the microdrive array system in freely moving mice.

INTRODUCTION:

It is crucial to understand how neuronal activity supports cognitive process, such as learning and memory, by investigating how different brain regions dynamically interact with each other. To elucidate dynamics of the neural activity underlying cognitive tasks, large-scale extracellular electrophysiology has been conducted in freely moving animals with the aid of microdrive arrays[1-4]. In the past two decades, several types of microdrive array have been developed to implant electrodes into multiple brain regions for rats[5-8] and mice[9-12]. Nonetheless, current microdrive designs generally do not allow for the use of multiple probe types, forcing researchers to choose a single electrode type with specific benefits and limitations. For example, tetrode arrays work well for densely populated brain regions such as the dorsal hippocampus CA1[1,13], while silicon probes give a better geometrical profile for studying anatomical connections[14,15].

Tetrodes and silicon probes are often used for in vivo chronic recording, and each has its own advantages and disadvantages. Tetrodes have been proven to have significant advantages in better single unit isolation than single electrodes[16,17], in addition to cost effectiveness and mechanical rigidity. They also provide higher yields of single unit activities when combined with microdrives[8,18-20]. It is essential to increase the number of simultaneously recorded neurons for understanding the function of neural circuits[21]. For example, large numbers of cells are needed to investigate small populations of functionally heterogeneous cell types such as time-related[22] or reward coding[23] cells. Much higher cell numbers are required to improve the decoding quality of spike sequences[13,24,25].

Tetrodes, however, have a disadvantage in recording spatially distributed cells, such as in the cortex or thalamus. In contrast to tetrodes, silicon probes can provide spatial distribution and interaction of local field potentials (LFPs) and spiking activities within a local structure[14,26]. Multi-shank silicon probes further increase the number of recording sites and allow recording across single or neighboring structures[27]. However, such arrays are less flexible in the positioning of electrode sites compared to tetrodes. In addition, complex spike sorting algorithms are required in high-density probes to extract information about action potentials of neighboring channels to mirror the data acquired by tetrodes[28-30]. Hence, the overall yield of single units is often less than tetrodes. Moreover, silicon probes are disadvantageous due to their fragility and high cost. Thus, the choice of tetrodes vs. silicon probes depends on the aim of the recording, which is a question of whether obtaining a high yield of single-units or spatial profiling at the recording sites is prioritized.

In addition to recording neural activity, optogenetic manipulation has become one of the more powerful tools in neuroscience to examine how specific cell types and/or pathways contribute to neural circuit functions[13,31-33]. However, optogenetic experiments require additional consideration in microdrive array design to attach the fiber connector to stimulation light sources[34-36]. Often, connecting fiber-optics requires a relatively large force, which may lead to a mechanical shift of the probe in the brain. Therefore, it is not a trivial task to combine an implantable optical fiber to conventional microdrive arrays.

For the above reasons, researchers are required to optimize the selection of the type of electrode or to implant an optical fiber depending on the aim of the recording. For example tetrodes are used to achieve higher unit yield in hippocampus[1,13], while silicon probes are used to investigate the laminar depth profile of cortical areas, such as the medial entorhinal cortex (MEC)[37]. Currently, microdrives for simultaneous implantation of tetrodes and silicon probes had been reported for rats[5,11]. However, it is extremely challenging to implant multiple tetrodes and silicon probes in mice because of the weight of the microdrives, limited space on the mouse head, and spatial requirements for designing the microdrive to employ different probes. Although it is possible to implant silicon probes without a microdrive, this procedure does not allow for adjustment of the probe and lowers the success rate of silicon-probe recovery[12,38]. Furthermore, optogenetic experiments require additional considerations in microdrive array design. This protocol demonstrates how to construct and implant a microdrive array for chronic recording in freely moving mice, which allows implantation of nine independently adjustable tetrodes and one adjustable opto-silicon probe. This microdrive array also facilitates optogenetic experiments and recovery of the silicon probe.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center.

1. Preparations of microdrive array parts

1.1. Print the microdrive array parts using a 3D printer using dental model resin (**Figure 1A,B**). Ensure that the thickness of individual 3D printed layers is less than 50 µm to keep the small holes on the printed parts clear and viable.

NOTE: The microdrive array consists of five parts (**Figure 1C**): (1) the main body of the microdrive array, which includes nine microdrive-screws for tetrodes and one screw for a silicon-probe (**Figure 1Ca-d**). The coordination of the tetrode bundle and hole for the opto-silicon probe at the bottom depends on the target brain area's coordinates (**Figure 1Cd**); (2) a shuttle to attach a silicon-probe or optrode (**Figure 1Ce**); (3) a probe electrical connecter mount to hold the silicon probe connecter (**Figure 1Cf**); (4) a fiber ferrule holder that clamps to the center part of the body to prevent undesired movements of the implanted opto-silicon probe when plugging/unplugging an optical fiber connector (**Figure 1Cg**); and (5) a shielding cone that

provides physical and electrical shielding to the microdrive array for stable recording (**Figure 1Ch**). The total weight of the microdrive array is 5.9 g, including the shielding cone (**Table 1**). If holes are clogged in the printed parts, drill out the holes using drill bits: #76 for the inner holes and #68 for the outer holes for tetrode-microdrive screws, #71 for tetrode microdrive-screw supporter hole, and #77 for the holes for the guide-posts at the bottom of the body.

1.2. Insertion of guide posts into the microdrive array body.

1.2.1. Cut two 16 mm lengths of 26-Ga stainless steel wire. Gently sharpen the wire tips using a rotary grinder.

1.2.2. Insert the wires into the bottom holes of the body (**Figure 2A**). Apply a small amount of cyanoacrylate glue at the bottom of the body to secure the guide posts.

2. Opto-silicon probe preparation

2.1. Prepare the microdrive screw for a silicon-probe.

NOTE: The microdrive screw for the silicon probe consists of a custom screw (300 µm pitch), supporting a support tube, and an L-shape tube (**Figure 2B**).

2.1.1. Prepare the mold for the microdrive head (**Figure 2C**). To construct the mold, prepare the 3D-printed plastic pattern of the microdrive (**Figure 2Ca**). Then, pour liquid silicone gel after making a temporal wall by putting tapes around the pattern. Remove air bubbles by shaking gently, wait until it is cured, then remove the silicone-gel mold from the pattern (**Figure 2Cb**).

2.1.2. Cut 18 mm and 9.5 mm lengths of 23 G stainless wire using a rotary grinder. Roughen the top 2–3 mm of the wires with a rotary grinder to enhance adhesion of the dental acrylic.

2.1.3. Take one custom screw and apply small amount of silicon oil to reduce the friction with the dental acrylic. Set the wires and a custom-screw to the mold.

2.1.4. Pour dental acrylic into the mold using a syringe to eliminate air bubbles around the wires and the screws. Air bubble contamination will make the microdrive fragile. Wait until the dental acrylic is fully cured, then take off the microdrive screws from the mold. Bend 6 mm of the longer wire tip to a 60° angle using pliers.

2.1.5. Check the quality of the microdrive screws (e.g., cracks, air-bubbles, and friction) to rotate the screw. If there is high friction, rotate the screw until they become smooth using an electric screw driver with a customized driver tip, which couples with the microdrive screw.

2.1.6. Install the microdrive screw into the microdrive array body to check whether it moves up and down smoothly by turning the screw. Threads for the screw are automatically created when inserting the screw into the hole of the body.

2.2. Prepare the shuttle (**Figure 3Aa**).

2.2.1. Cut two 5 mm lengths of polyetheretherketone (PEEK) tubing using sharp scissors. Align the tubes at both sides of the shuttle. Glue the tubes and shuttle using epoxy.

2.2.2. Apply small amount of silicon oil on the guide posts. Check the quality of the shuttle by inserting onto the guide posts of the microdrive array body. Make sure the shuttle moves smoothly without excessive friction.

2.3. Prepare an optorode (**Figure 3Ab**). This step can be skipped if an optogenetic experiment is not required.

2.3.1. Cleave the optical fiber to 21 mm in length using a ruby cutter. Grind the fiber tip to make the tip flat and shiny.

2.3.2. Gently place the optical fiber on the front side of the silicon-probe. The fiber tip is positioned 200–300 µm above the top of the electrode sites. Hold the fiber temporarily with transparent tape.

2.3.3. Glue the optical fiber to the base of the silicon-probe using small amount of epoxy. Wait for at least 5 h until the epoxy is fully cured.

NOTE: It is recommended to attach the optical fiber on the same side as the electrode sites. Attaching the fiber at the backside may prevent light from properly illuminating the recording sites.

2.4. Attach the shuttle to the silicon probe (**Figure 3Ac**): apply a small amount of epoxy at the back of the silicon-probe's base. Attach the bottom part of the shuttle to the silicon-probe's base, and gently hold in position for 2–3 min to avoid formation of a gap between the shuttle and silicon-probe base during initial cure of the epoxy. Wait for at least 5 h until the epoxy is completely cured.

2.5. Carefully insert the shuttle tubes onto the guide posts of the main body under the microscope (**Figure 3B**). During this procedure, hold the groove of the shuttle with fine tweezers.

2.6. Insert the microdrive-screw into the screw hole by turning the screw. Engage the silicon probe and microdrive-screw by inserting the tip of the L-shape wire into the groove of the shuttle head (**Figure 3C**).

2.7. Attach the probe electrical connecter holder to the microdrive array body (**Figure 3D**).

2.7.1. Cut two #0 screws to 3.5 mm thread length. Grind the tips to remove burrs.

2.7.2. Place the probe connecter holder on the body. Place the silicon probe electrical connector into the holder.

2.7.3. Secure the silicon probe connector in the holder using epoxy, and be sure to not glue it to the microdrive array body to allow for the recovery procedure of the silicon probe. Insert the screws to hold the probe connecter holder.

2.8. Attach the ferrule-holder to the opto-silicon probe and microdrive array body (**Figure 3D**).

2.8.1. Cut two #0 screws to 6 mm thread length. Grind the tips to remove burrs.

2.8.2. Grind the outside of two #0 machine screw nuts to make small hex nuts with 2.5–3.0 mm outer diameter to reduce the weight and space.

2.8.3. Insert the screws into component A of the holder. Glue the screw heads using epoxy.

2.8.4. Apply small amount of silicon grease to component A and B to reduce friction with the body. Insert component A into the body, then temporally hold using inverse tweezers.

2.8.5. Place component B onto component A's screws. Thread the customized nuts into the screws. Use pliers to tighten the nuts to secure the ferrule holder on the body.

2.8.6. Insert the fiber ferrule into the groove of the fiber ferrule holder (component B). Ensure that the fiber ferrule is sticking 4–5 mm out from the holder.

2.8.7. Apply small amount of epoxy between the ferrule and the holder groove. Wait until the epoxy is fully cured and check that the ferrule does not move. Check the shuttle and ferrule holder for smooth motion by loosening the nuts before turning the microdrive-screw.

2.8.8. Check the working distance of the probe. Ensure that the probe tip completely retracts into the body when the ferrule-holder is at the top position while the shuttle tubes are still associated with the guide-posts. The maximum working distance is determined by the length of the silicone probe and the target brain region.

2.8.9. If the microdrive-screw is loose, apply small amount of dental acrylic around the screw to add more threads for support. When it is cured, rotate the screw to check tightness and stability.

3. Tetrode preparation

NOTE: This procedure is similar to previously published articles[8,19,20,39].

3.1. Prepare the microdrive screws for the tetrode. The microdrive for a tetrode consists of a

custom-machined screw and a 23 G tubing (**Figure 2B**). This procedure is similar to section 2.1.

3.2. Make a bundle of 30 G stainless steel tubing that has a 5.5 mil wire inside. In this case, a total of nine 30 G tubing (eight recording tetrodes and one reference electrode) were used.

3.3. Thread the 30 G bundle from the bottom of the drive body, and secure them with 20 G thin-walled tubing to the main body. Trim the bottom of the bundle with a rotary grinder to make the tip even and flush. Trim top part of the 30 G tubes with a rotary grinder so that the 30 G tube sticks out about 0.5 mm from the main body.

3.4. Load 5.5 mil polyimide insulating tubes into the 30 G tubing. Prepare tetrode wires and load them into a 32-channel electric interface board (EIB). Check electrical connection with the impedance tester before final precision cut.

3.5. Lower electrode tip impedance to 250–350 kΩ with gold plating solution. Fix all tetrodes with superglue.

3.6. Fill excessive gap between polyimide tube and tetrode with mineral oil for sealing and lubrication. Route the ground wire to the EIB.

NOTE: If necessary, the optical fiber can be integrated along tetrode wires[12].

4. Attaching the shielding cone

4.1. Paint silver conductive shielding paint on the inside of the printed cone. Place the microdrive array inside of the cone (**Figure 3E**).

4.2. Cut two #0 screws to 3.5 mm thread length. Fasten the screws from the outside of the cone to hold the microdrive array in place.

4.3. Apply silver paint around the screw head to electrically connect the shielding cone with electrical ground. Check the electrical connectivity between the ground wire and cone. Apply a small amount of epoxy between the microdrive array body and shielding cone to securely attach the body.

NOTE: Another way to prepare the shielding cone is to use aluminum tape[40] (**Figure 3F**). First, prepare the pattern paper for the shielding cone after sticking aluminum foil to the paper (**Figure 3Fa**). Then, roll the paper and attach it to the microdrive body using a small amount of cyanoacrylate glue (**Figure 3Fb**). The weight of this cone is 0.72 g and total weight of the microdrive array is reduced to 4.7 g (**Table 1**).

5. Implant surgery

NOTE: This procedure is modified from previously published articles[18,39,41] for dual-site

implantation. Ensure that the weight of the animal is over 25 g for the microdrive implant for faster recovery after the surgery.

5.1. Preparation

5.1.1. To prepare a ground screw, attach the silver wire to a skull screw and apply silver paint. Then, attach a gold pin to the opposite side of the wire using silver paint.

5.1.2. Prepare the drive holding adapter to hold the microdrive array to a stereotactic device. Attach a male connecter to a stainless handle using epoxy. Make sure that alignment of the connecter and stainless handle is straight.

5.1.3. In the case that histological confirmation is needed after recording, apply Di-I to the tetrodes or backside of the silicon-probe[38].

5.1.4. Lower the silicon-probe down to be desired depth. Loosen the nuts of the ferrule holder using pliers, lower the silicon-probe (opto-silicon probe) by turning the silicon-probe's microdrive-screw, then fasten the nuts to secure the ferrule holder. When implanting tetrodes in hippocampal area CA1 and a silicon-probe in MEC, the distance between the tetrode cannula and silicon-probe's tip is 3–4 mm.

5.2. Set the anesthetized mouse (0.8%–1.5% isoflurane) in a stereotaxic device. The anesthetic condition of the mouse is confirmed by absence of the toe-pinch reflex. Apply clear ointment to the eyes to prevent drying. Cover the eyes with a piece of foil to protect from strong surgical light exposure.

5.3. Disinfect the mouse's scalp with iodine and isopropanol after shaving the fur. Make a 1.5–2.0 cm incision at the scalp using standard surgical scissors, and remove the tissue over the skull using cotton swabs after subcutaneously applying lidocaine.

5.4. Align the mouse head with the stereotaxic tool. Ensure that the height difference between bregma and lambda is less than 100 μm. Determine the craniotomy location using an atlas and mark these locations with a sterilized pencil.

5.5. Anchor the skull screws (0.8 mm diameter, 0.200 mm thread pitch) by rotating them 1.5 turns (0.3 mm) on the skull, using surgical tweezers and a screwdriver after drilling 8–11 holes in the skull using 0.5 mm drill bit.

NOTE: 2–4 holes in the frontal skull, 2–3 holes in each side of the parietal skull, and 1–2 holes in the interparietal skull are suggested.

5.6. Attach the ground screw to the hole by rotating it one turn (0.2 mm) after drilling a hole in the interparietal bone. Ensure that this hole does not penetrate through the bone into the brain case; otherwise, cerebellar signals will contaminate the recording. Check that the impedance is

less than 20 kΩ at 1 kHz between the ground screw and skull screws using an impedance meter.

NOTE: Larger impedance will cause the introduction of motion artifacts during recording.

5.7. Perform the craniotomy at the marked locations. The dura can be left intact in mice.

5.8. Connect the male pin of the ground screw and the microdrive array's ground connector. Check connectivity using the impedance meter by measuring between the ground screw and shielding.

5.9. Set the microdrive array to the adapter, set it to the stereotaxic device, and slowly lower the silicon probe until the desired depth. Ensure that the tetrode bundles are placed above the brain surface but still inside of the microdrive array when the silicon probe is inserted into the brain (**Figure 4A**).

5.10. Carefully apply the silicon grease to seal the area of the silicon probe and the tetrode bundle (**Figure 4B**). Put a small amount of the silicon grease at the tip of a 20 G needle and apply the grease around the probes using the needle. Repeat until silicon grease completely covers the area around the probe so that dental acrylic does not flow onto or underneath the electrodes/probes. Be careful not to let the grease touch the electrode sites, otherwise it will dramatically increase the impedance of the recording sites.

5.11. Apply dental acrylic to fix the microdrive array to the anchoring screws in the skull.

NOTE: It is recommended to apply dental acrylic in three layers to avoid the excessive heat produced during curing of the acrylic.

5.12. Remove the adapter from the microdrive array carefully. Inject 1 mL of PBS subcutaneously to prevent dehydration. Inject 5 mg/kg meloxicam subcutaneously as an analgesic treatment.

5.13. Cover the silicon-probe connector by a piece of tape to prevent any dirt from getting inside of the electrical connections. Cover the microdrive array using a plastic paraffin film and tape it in place.

5.14. Administer appropriate analgesic treatment for 3 days (e.g., subcutaneous injections of 2 mg/kg meloxicam once per day). Allow 3–5 days for recovery before starting the tetrode adjustment. The implanted mouse after the recovery period is shown in **Figure 4C**.

6. Recovering the silicon-probe (**Figure 4D**)

6.1. Inject ketamine (75 mg/kg) and dexmedetomidine (1 mg/kg) anesthetics intraperitoneally and confirmed absence of the toe-pinch reflex. Fix the anesthetized mouse by directly perfusing 4% paraformaldehyde through the heart using a hood. Surgical methods for rodents are

described previously[42].

6.2. Loosen the nuts of the ferrule holder using a plier. Then, carefully move it to the top of the body by turning the adjusting screw to fully retract the silicon-probe towards inside of the microdrive array body. Fasten the nuts to hold the probe at the top position.

6.3. Take the mouse brain out from the bottom by cracking the skull from the side. The microdrive array is now separated from the animal.

6.4. Completely remove the L-shaped microdrive-screw that drives the silicon-probe. Loosen and take out the nuts of the ferrule holder using pliers. Take out the component A of the ferrule holder.

6.5. Unscrew the probe connector mount and detach from the drive body. Check that the probe connector mount can come off from the microdrive array body.

6.6. Hold the top part of the shuttle with tweezers, then carefully slide the silicon-probe assembly out from the microdrive array.

6.7. Clean the probe tip with contact lens cleaner (first with enzyme, then 3% hydrogen peroxide) for at least 1 day. Carefully wipe the electrode tip using isopropanol pads under the microscope. Keep the probe in a static-free storage box.

NOTE: The shuttle and probe connector mount remain attached to the silicon probe and can be reused in the next implantation.

6.8. To reuse the microdrive array body for the next surgery, remove the dental acrylic using a combination of fine-tip drills and nippers. Then, recover the skull-screws by immersing the removed dental acrylic into acetone. Note that the acetone will dissolve plastic parts of the microdrive array.

6.9. Remove the epoxy between the microdrive body and shielding cone using a scalpel.

NOTE: No additional parts need to be printed again for the next surgery if the microdrive is not broken.

REPRESENTATIVE RESULTS:

The microdrive array was constructed within 5 days. The timeline of microdrive preparation is described in **Table 2**. Using this microdrive, nine tetrodes and one silicon probe were implanted into the hippocampal CA1 and MEC of the mouse [21 week old/29 g body weight male pOxr1-Cre (C57BL/6 background)], respectively. This transgenic mouse expresses Cre in MEC layer III pyramidal neurons. The mouse was injected with 200 nL of AAV5-DIO-ChR2-YFP (titer: 7.7 x 10^{12} gc/mL) into the MEC 10 weeks before the electrode implant. LFPs were recorded using a low-

pass filter (1–500 Hz), and spiking units were detected using a high-pass filter (0.8–5 kHz). Light stimulation (λ = 450 nm) was performed using a 1 ms pulse width at 10.6 mW intensity measured at the end of the fiber connector. The reference electrode for the tetrode recording was placed in the white matter using a dedicated tetrode wire. The reference for the silicon probe recording was set as the top channel of the probe.

After the tetrode adjustment, behavioral performance was tested on a linear track (**Figure 5A**) and in an open field (**Figure 5B**). In both experiments, the mouse explored freely for ~30 min (**Figure 5Aa,b,c; Figure 5Ba,b,c**). The electrophysiological signals were successfully recorded without severe motion-related noise throughout the recording session (**Figure 5Ad,e; Figure 5Bd,e**). Next, light stimulation was performed at the MEC to stimulate MEC layer III neurons that project to the CA1[43] (**Figure 6A**). Spontaneous spiking activities (**Figure 6B,C**) and LFPs (**Figure 6D**) were recorded from the tetrodes and silicon probe when the mouse was sleeping. LFPs recorded in the tetrodes showed large ripple activities, suggesting that all tetrodes were positioned in the vicinity of the CA1 pyramidal cell layer. Light-induced responsive activities were first observed in MEC, followed by in CA1 with 13–18 ms latency (**Figure 6E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Microdrive array overview. (A) A skeleton view of the microdrive array, from the tetrode side (a) and silicon-probe side (b). **(B)** A real image of the loaded microdrive array, viewed from the tetrode side (a) and from the silicon-probe side (b). The microdrive array is placed on the jig stage in panel (b). **(C)** Individual 3D-printed microdrive array parts. (a–d) The microdrive array body, viewed from four different angles (a: tetrode side view; b: silicon-probe side view; c: top view; d: bottom view). A magnified view of the dashed line in panel (c) is shown in **Figure 2A**. (e) The shuttle, which holds and allows adjusting of the silicon-probe. A silicon probe is attached at the dashed line in panel (e). (f) The probe-connecter holder, which holds a 32-channel silicon-probe connecter. (g) The fiber ferrule holder, which holds an optical fiber ferrule to prevent from the movement of the probe when plugging/unplugging the fiber connector with the light-source. This part consists of two components: [panel (g) and components A and B]. (h) The printed shielding cone, which provides physical and electrical shielding when painted with conductive material. The cone window allows the ability to see inside of the structure during microdrive array preparation, which is eventually covered by a piece of tape or 3D-printed material.

Figure 2: **Preparation of guide-posts and microdrive screws on the main body**. **(A)** Guide post-preparation. (a) Magnified view of the microdrive array body shown in **Figure 1Cc**. (b) Guide post-insertion into the holes of the body. **(B)** The microdrive-screw designs. (a) The microdrive-screw for a silicon-probe, which consists of a 300 µm pitch custom screw, supporting tube, and L-shape tube. (b) The microdrive-screw for a tetrode, which consists of a 160 µm pitch custom screw and 30 G stainless guide tube. **(C)** Fabrication of the top piece of the microdrive-screws: (a) Preparation of 3D-printed patterns of the anti-mold for the microdrive-screw. The picture shows a pattern for the silicon-probe microdrive-screw. (b) The mold made using the anti-mold pattern (a) and silicon-rubber material. Assembled microdrive-screws are produced by inserting

custom-screws and wires/tube, and pouring dental acrylic in each well. Inset: magnified view of the wells of the mold.

Figure 3: Microdrive array assembly. (A) Preparation of an opto-silicon probe. (a) Attaching two plastic guide tubes to the shuttle. (b) Gluing the optical fiber to the silicon-probe. (c) Attaching the shuttle to the opto-silicon probe. In this picture, the bottom part of the shuttle (dashed line) is attached to the silicon-probe's base [backside of (b)]. The shuttle and the silicon probe shank should be in parallel. **(B)** Loading the opto-silicon probe shuttle assembly into the guide-posts of the microdrive array body. **(C)** Relative position of the silicon-probe microdrive when the probe is completely retracted into the body (a) and when positioned at the lowest in the drive body (b). The L-shape wire is inserted into the groove on the shuttle. **(D)** An exploded view of the fiber ferrule holder and the probe connector mount. **(E)** Shielding cone attached. The conductive material is painted inside of the cone. **(F)** Alternative shielding cone using a paper and aluminum tape. (a) A pattern paper. (b) An attached alternative shielding cone, which reduces 1.1 g of weight compared to the 3D-printed version.

Figure 4: Sealing the probes during surgery and recovery of the silicon probe. (A) The microdrive array and the mouse skull after craniotomy, before applying silicon-grease. The silicon-probe is inserted about 2mm into the brain at this time. **(B)** Applying silicon-grease around the silicon-probe and tetrode bundles to protect the probes from dental acrylic. **(C)** The chronically implanted mouse after the recovery period, when the mouse is walking (a), grooming (b), and when connected to the recording cable with the counter-balancing pulley system (c). **(D)** The recovered silicon probe, before (a) and after (b) immersion into the cleaning solution. The biological tissues in (a) are removed after the cleaning process (b).

Figure 5: Examples of simultaneous tetrode/silicon-probe recording in the hippocampal CA1 and medial entorhinal cortex (MEC) from the behaving mouse. (A) Recording on the linear track. (a) The linear track used for the recoding. (b) Trajectories of the mouse exploration for ~30 min on the track. (c) Behavioral performance on the linear track. (d-e) Representative LFP recordings from the tetrode (d) and the silicon-probe (e). **(B)** Recording in the open field. (a) The open field chamber used for the recoding. (b) Trajectories of the mouse exploration for ~30 min in the chamber. (c) Behavioral performance in the open field. (d,e) Representative LFP recordings from the tetrode (d) and the silicon-probe (e). LED is attached to the head amplifier to record the positions of the mouse. The linear track and the open-field chamber are connected with the electrical ground to reduce electrostatic noise.

Figure 6: Representative results of simultaneous recordings in the CA1 and MEC and optogenetic stimulation. (A) Expression of AAV5-DIO-ChR2-YFP after 4 weeks of injection. MEC layer III pyramidal neurons that project their axons from dorsal MEC to dorsal CA1. Dashed lines: ori, stratum oriens; pry, stratum pyramidale; rad, stratum radiatum; mol, stratum lacunosum moleculare. **(B)** Representative spike recording from one of the tetrodes. (a) 2D cluster projections of spikes recorded from the tetrode. (b) Examples of the average spike waveform of three clusters, which are indicated by dashed lines in (a). **(C)** Representative spike recording from one of the silicon-probe electrode sites. (a) 2D cluster projections of spike

principal components. (b) Examples of the average spike waveform of three clusters. Spike clusters (pink and green) are separated from the noise clusters (blue). The clusters in (B,C) are calculated using KlustaKwik software. **(D)** Traces of spontaneous LFPs simultaneously recorded from the tetrodes in CA1 (a) and the silicon probe in MEC (b). Black arrows indicate the tetrode shown in (B) and silicon-probe electrode site shown in (C). **(E)** LFP responses to pulsed optical stimulation (10.6 mW, 1 ms; filled red arrowhead) from the tetrodes in CA1 (a) and silicon probe in MEC (b).

Table 1: Individual weight of each microdrive array part. The total weight of the microdrive array was 5.9 g after fixing the protective cone with epoxy (*in the case of using an alternative shielding cone using a paper and aluminum tape).

Table 2: The timeline of the microdrive preparation. The 3D-parts printing, waiting for curing the silicone rubber/dental acrylic/epoxy, and loading the tetrode wires take the majority of the time of the microdrive array preparation, in total 4–5 days.

Supplementary Files: The supplementary files include 3D model data of five microdrive parts in both .sldprt and .stl format. The original 3D model files were created with the software Solidworks2003.

DISCUSSION:

The protocol demonstrates how to construct and implant a hybrid microdrive array that allows recording of neural activities from two brain areas using independent adjustable tetrodes and a silicon-probe in freely behaving mice. It also demonstrates optogenetic experiments and the recovery of the silicon probe after experiments. While adjustable silicon probe[33] or opto-silicon probe[36] implantation are previously demonstrated in mice, this protocol has clear advantages in the simultaneous tetrode array and opto-silicon probe implantation to provide flexible choice of implanted probe types. The type of implanted probe can be switched depending on the aim of the experiment, such as multi-shank probes[27,44] or ultra-density Neuropixels[21,45]. The coordination and angle of implantation[7] can be easily modified at the 3D object design stage as needed. For example, dual-site or even triple-site recording is possible during learning tasks across memory-related brain structures, such as the hippocampus[46], entorhinal cortex[47], prefrontal cortex[48], amygdala[49], and cingulate cortex[50].

There are several critical procedures for successful implant and recording. Due to the fragility of silicon-based probes, any mechanical vibrations or impacts to the microdrive array should be minimized during assembly. For example, opening the clogged holes using a drill should be finished before loading the silicon probe into the microdrive array. Also, it should be emphasized to carefully check the ground connection in each step during microdrive array construction and implant surgery to ensure stability of the recorded data. Unstable or high-impedance connections to the ground cause heavy noise and motion related artifacts during the recording session. For stable recordings, it is recommended to wait 1–2 weeks after the surgery to avoid electrode drift because the brain tissue is negatively affected by the implant

surgery. However, signal quality on the silicon probe recovers after 1–2 weeks from the surgical trauma based on previous experience. It is recommended to use single-housing to prevent damage to the implanted microdrive array by other mice. For the optogenetic experiment, it is important to note that most silicon-probes induce photo-artifacts in response to light-stimulations[51], while others are designed to minimize photo-artifacts[52] (there are photo-artifact reduced silicon-probes that are commercially available).

The weight of the microdrive array (5.9 g) is heavier than the typical microdrives described in previous articles[12,53], mainly due to the microdrive array body (~21% of the total weight), shielding cone (~31%), and metal parts (screws and nuts: ~22%). It is recommended to use mice with weights of over 25 g (~2–3 months old for C57BL/6 mice[54,55]) for implant surgery, because mice with adequate body weights tend to recover earlier. For this reason, this microdrive array may be not the best solution for juvenile mice. While devices that are 5%–10% of the mouse's bodyweight are often guided to be tolerated for implants[12,56] (although there is no supporting published data for this[57]), this microdrive array weighs ~24% of the bodyweight of 25 g mice (~19% when using the alternative cone described below).

However, the implanted adult mice were able to freely move around and jump around in the home cages. Mice implanted with a similar microdrive array weight (~4.5 g) have previously been shown to perform the behavioral task (linear maze task) even under food restriction[13,17]. The disadvantage of weight is not a problem during recording, as a counterweight balancing system[18,34,58] or headpost system[59] will support the microdrive array. In addition, the total weight of the microdrive array can be reduced by lowering the height or reducing the thickness of the shielding cone and modifying the design to utilize smaller screws.

Using the current 3D printing material, the thickness of the shielding cone can be reduced up to ~0.3 mm (from the current thickness of ~0.6 mm). The cone height can be reduced ~5 mm as long as the tetrode wires can still be covered. Exposure of the tetrode wires will result in breakage of the wires and failure of the long-term recording. Alternatively, preparation of the shielding cone using paper and aluminum tape can reduce the cone weight to ~0.7 g (~15% of total weight; reduced 20% from the total weight of the original microdrive array); although, these are a tradeoff with the physical strength. In addition, the size of the microdrive (current shielding cone: 4.2 x 4.0 x 2.6 cm = major axis x minor axis x height) can be an obstacle to food and water access if they are provided from the top of the animal cage. As long as they are provided on the cage floor or from the sidewall, the microdrive does not disturb natural behaviors of mice, such as eating, drinking, grooming, rearing, or nesting[60].

In conclusion, this microdrive protocol provides researchers with flexible choices for recording from multiple brain areas in freely moving mice for understanding the dynamics and functions of long-ranging neural circuits.

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The authors have nothing to disclose.

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Figure 1

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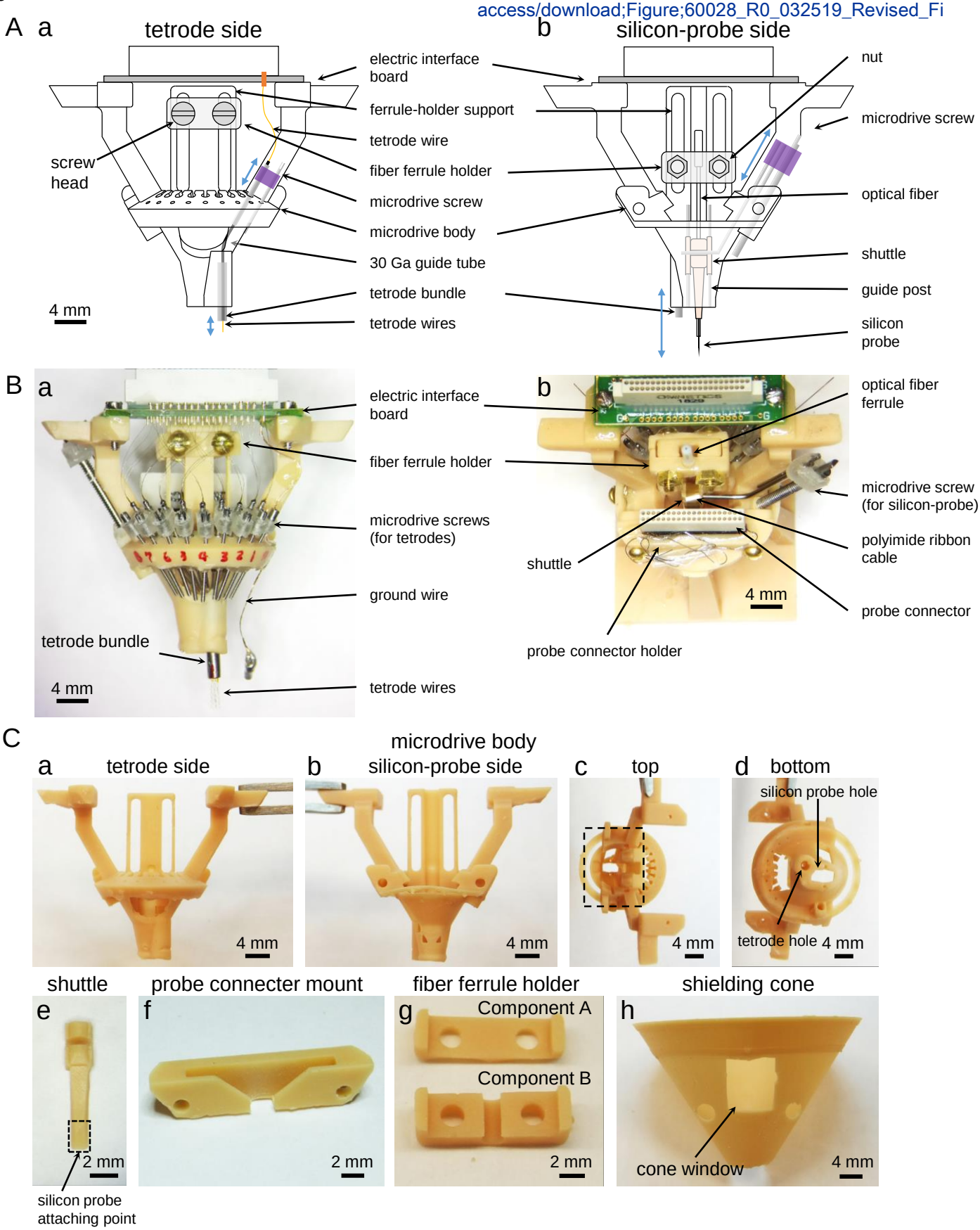
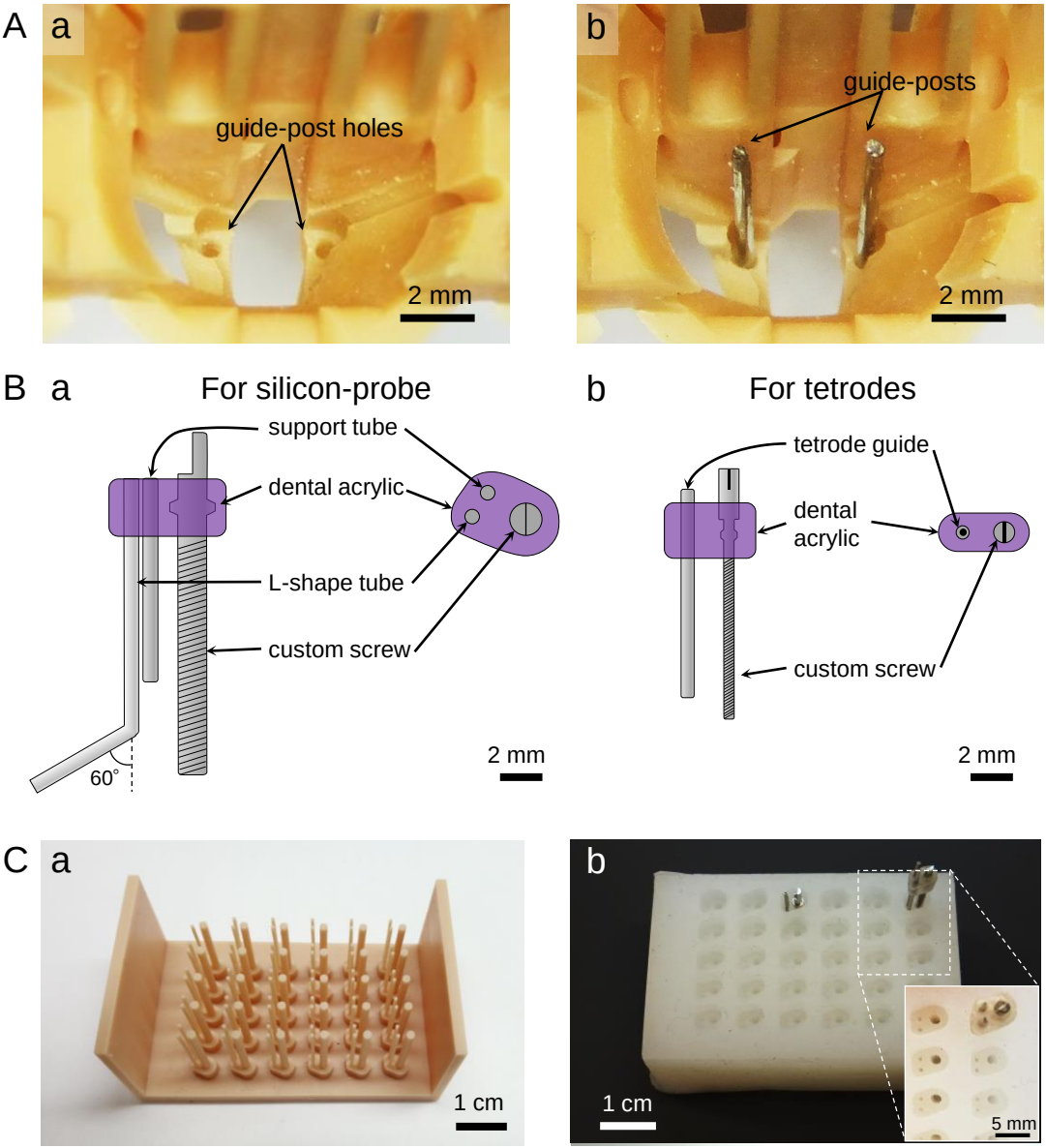
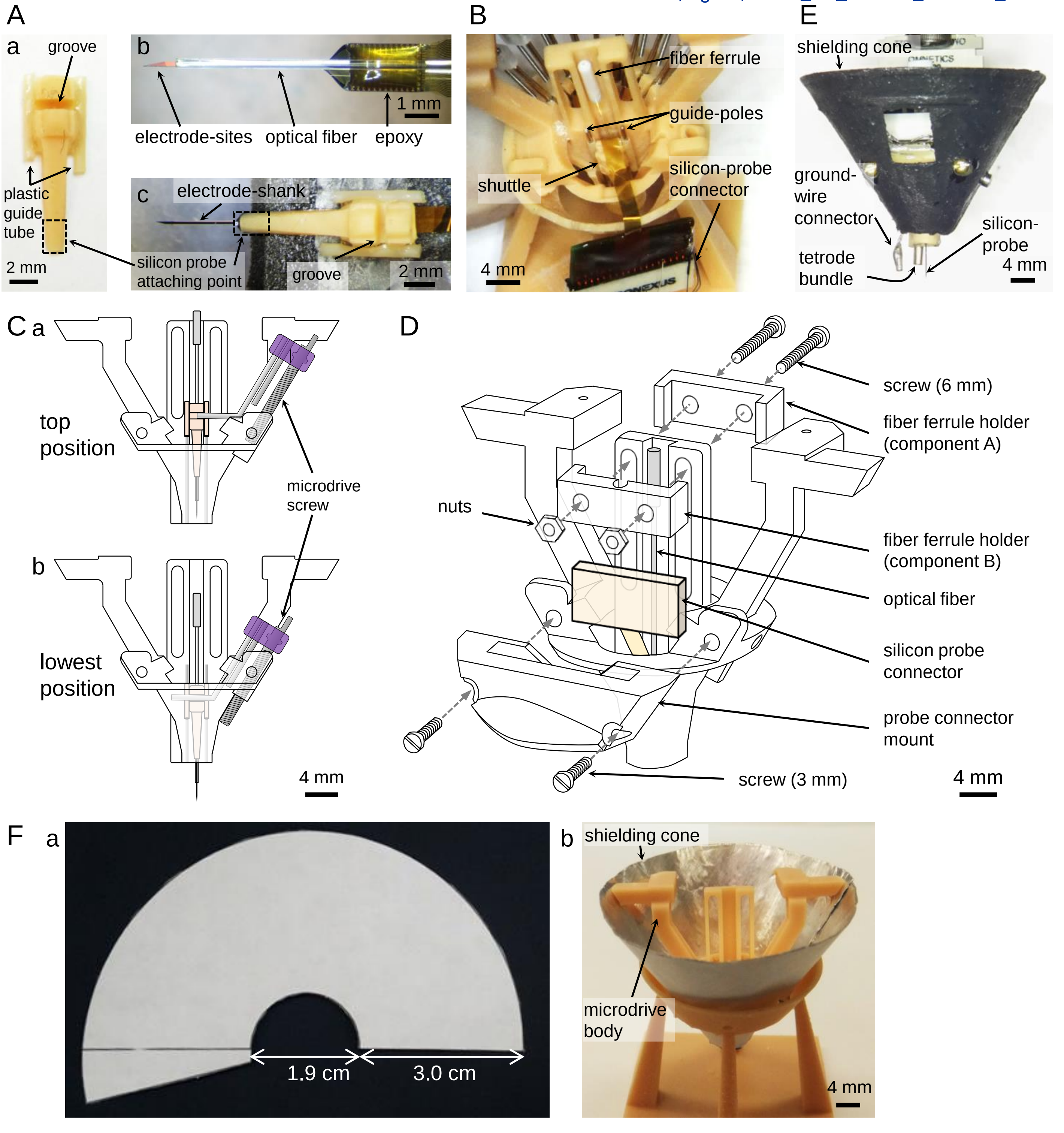
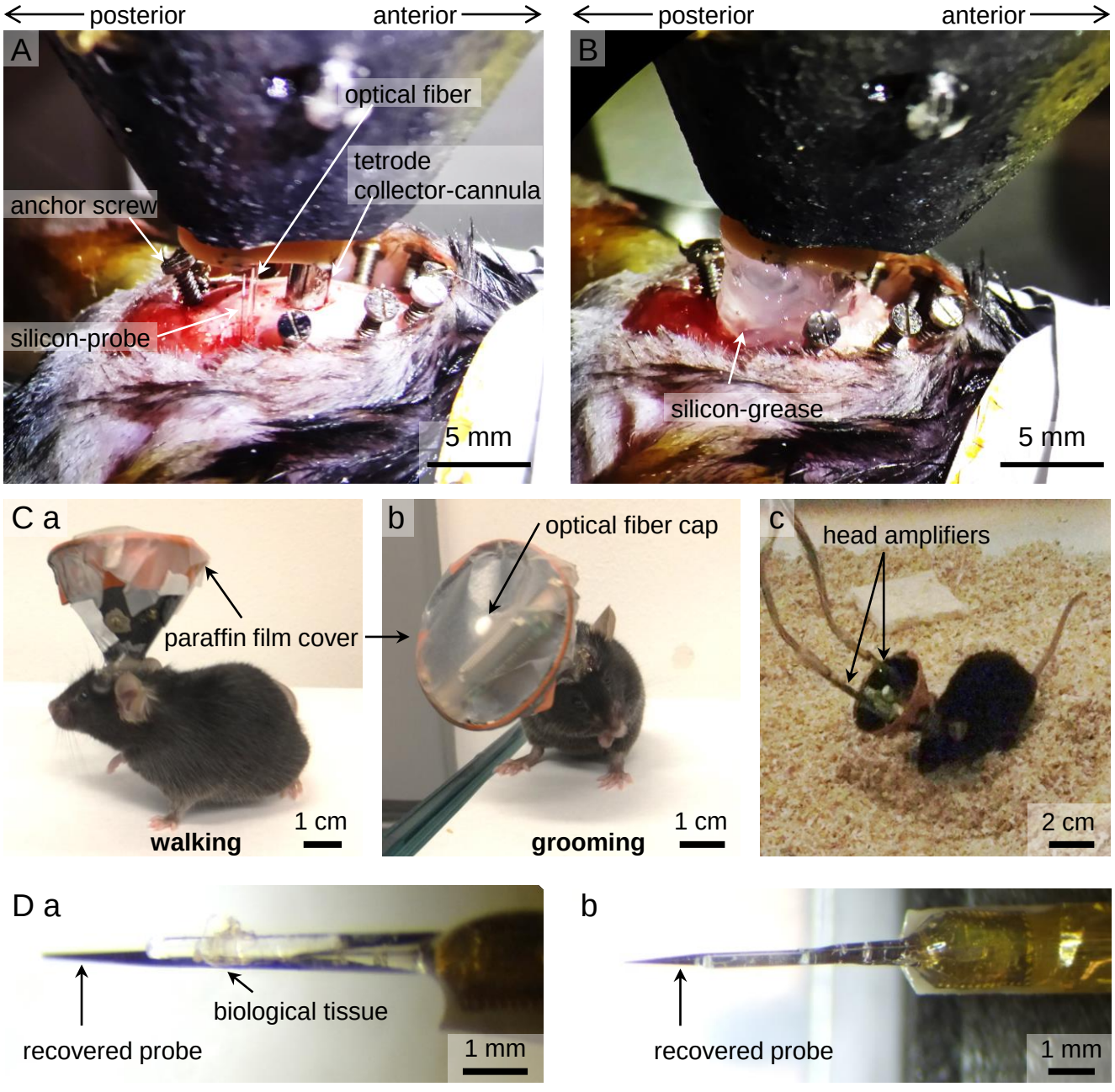



A
a
guide-post holes
2 mm
b
guide-posts
2 mm
B
a
For silicon-probe
support tube
dental acrylic
L-shape tube
custom screw
60°
2 mm
b
For tetrodes
tetrode guide
dental acrylic
custom screw
2 mm
C
a
1 cm
b
1 cm
5 mm


A
a
groove
plastic guide tube
2 mm
b
1 mm
electrode-sites
optical fiber
epoxy
c
electrode-shank
silicon probe attaching point
groove
2 mm
B
fiber ferrule
guide-poles
shuttle
silicon-probe connector
4 mm
E
shielding cone
ground-wire connector
tetrode bundle
silicon-probe
4 mm
C a
top position
microdrive screw
b
lowest position
4 mm
D
screw (6 mm)
fiber ferrule holder (component A)
nuts
fiber ferrule holder (component B)
optical fiber
silicon probe connector
probe connector mount
screw (3 mm)
4 mm
F
a
1.9 cm
3.0 cm
b
shielding cone
microdrive body
4 mm


posterior
anterior
A
optical fiber
tetrode
collector-cannula
anchor screw
silicon-probe
5 mm
posterior
anterior
B
silicon-grease
5 mm
C a
paraffin film cover
1 cm
walking
b
optical fiber cap
1 cm
grooming
c
head amplifiers
2 cm
D a
biological tissue
recovered probe
1 mm
b
recovered probe
1 mm

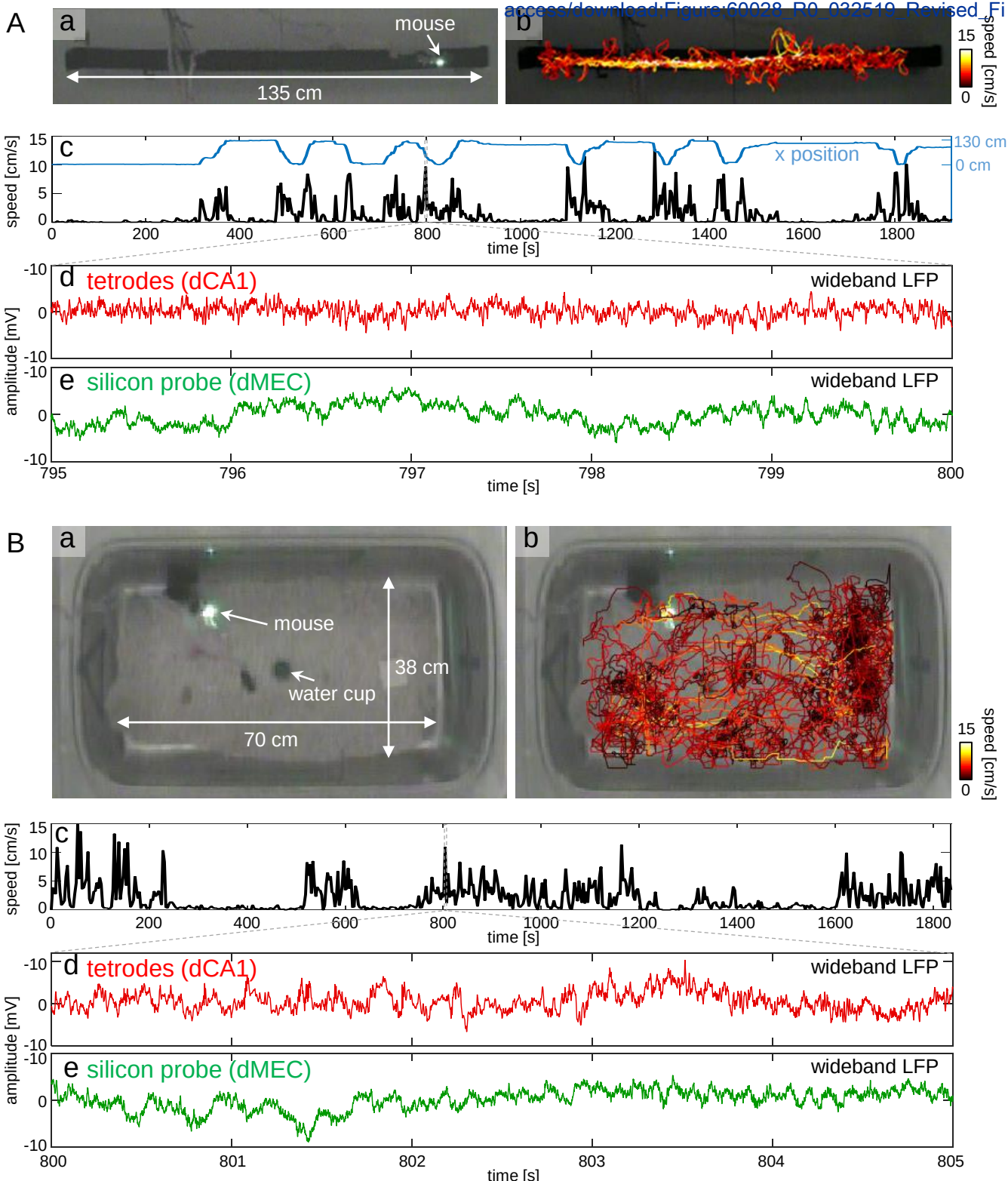
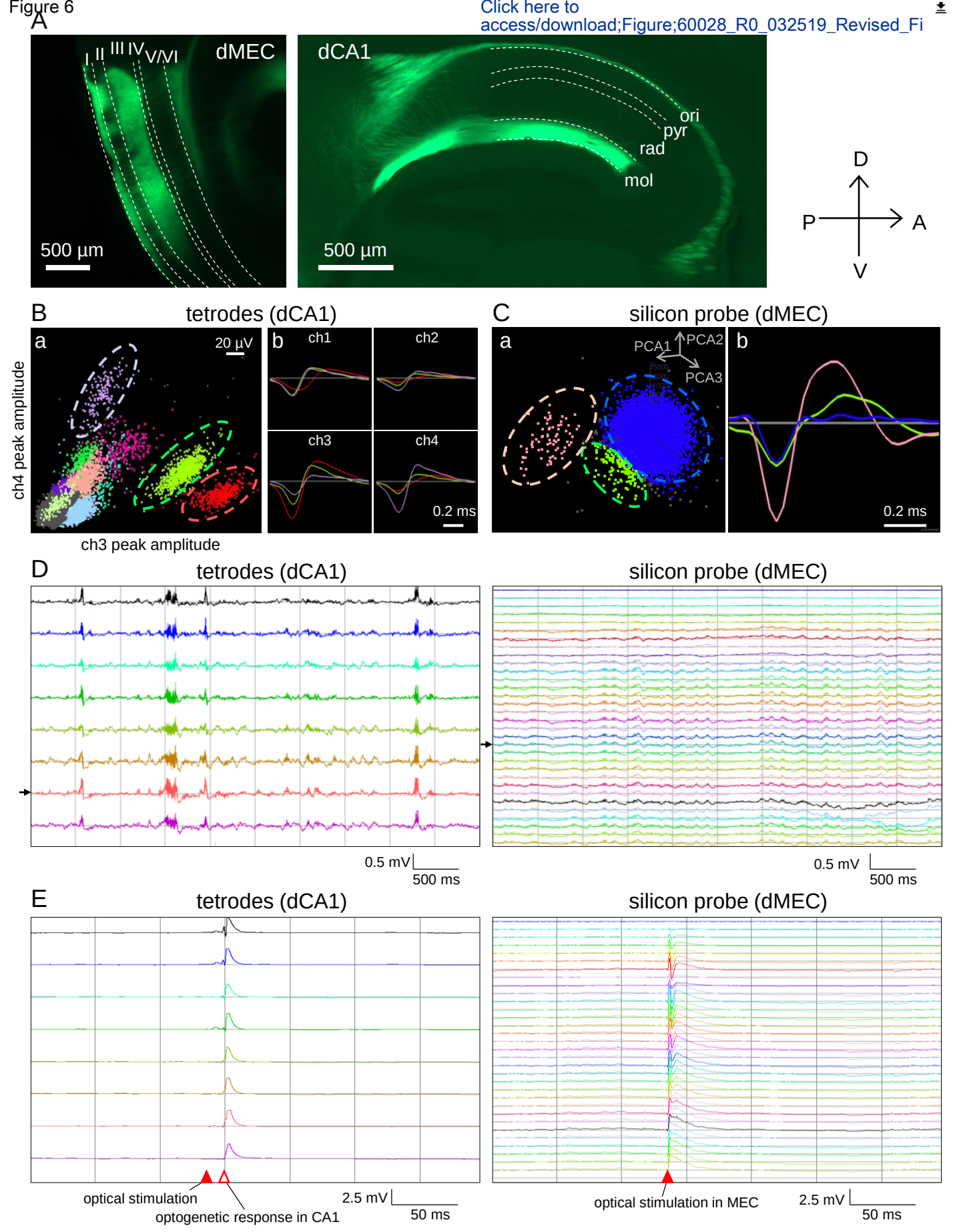


Figure 6



Revised Table 1

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	grams/one	number	sum [gram]	
main body	1.25	1	1.25	
shuttle	0.04	1	0.04	
probe connecter mount	0.19	1	0.19	
fiber ferrule holder	0.1	1	0.1	
shielding cone	1.82	1	1.82	(0.72)*
conductive paste	0.2	1	0.2	
machine screw (#00, 2 mm), to ho	0.05	2	0.1	
machine screw (#0-80, 3.5 mm)	0.06	4	0.24	
machine screw (#0-80, 6mm)	0.09	2	0.18	
nut	0.03	2	0.06	
microdrive (tetrode)	0.05	9	0.45	
microdrive (silicon probe)	0.29	1	0.29	
silicon probe	0.28	1	0.28	
electric interface board	0.6	1	0.6	
total			5.8	(4.7)*

procedures	time
microdrive preparation	
3D parts printing	1 day
optrode preparation	
Prepare the mold for the microdriv	1 day*
Microdrive head preparation	3 h
Attaching an optical fiber	3 h
Attaching a shuttle	3 h
tetrode preparation	
Prepare the mold for the microdriv	1 day*
Microdrive heads preparation	3 h
Loading tetrode wires	1 day
Attaching the shielding cone	
Painting shielding paint	overnight*
Attaching to the microdrive body	3 h

* these procedure can be conducted in parallel

Name of Material/Equipment	Company	Catalog Number	Comments/Description
#00-90 screw	J.I. Morris	#00-90-1/8	EIB screws
#0-80 nut	Small Parts	B00DGB7CT2	brass nut for holding fiber ferrule holder
#0-80 screw	Small Parts	B000FMZ57G	brass machine screw for
22 Ga polyetheretherketone tubes	Small Parts	SLPT-22-24	for attaching to the
23 Ga stainless tubing	Small Parts	HTX-23R	for tetrode
23 Ga stainless wire	Small Parts	HTX-23R-24-10	for L-shape/support wire
26 Ga stainless wire	Small Parts	GWX-0200	for guide-posts
30 Ga stainless wire	Small Parts	HTX-30R	for tetrode
3-D CAD software package	Dassault Systèmes	SolidWorks 2003	
3D printer	FormLab	Form2	
5.5mil polyimide insulating tubes	HPC Medical	72113900001-012	
aluminum foil tape	Tyco	Tyco Adhesives 617022 Aluminum Foil Tape	for the alternative shielding cone
conductive paste	YSHIELD	HSF54	for shielding cone
customized screws for silicon-probe microdrive	AMT	UNM1.25-HalfMoon	half-moon stainless screw, 1.5 mm diameter, 300 µm thread pitch
customized screws for tetrode microdrive	AMT	Yamamoto_0000-160_9mm	slotted stainless screw, 0.5 mm diameter, 160 µm thread pitch, custom-made to order for our design
dental acrylic	Stoelting	51459	
dental model resin	FormLab	RS-F2-DMBE-02	
Dremel rotary tool	Dremel	model 800	a grinder

drill bit	Fine Science Tool	19007-05	
electric interface board	Neuralynx	EIB-36-Narrow	
epoxy	Devcon	GLU-735.90	5 minutes epoxy
eye ointment	Dechra	Puralube Ophthalmic Ointment	to prevent mice eyes from drying during surgery
fiber polishing sheet	Thorlabs	LFG5P	for polishing the optical fiber
fine tweezers	Protech International	15-368	for loading/recovering the silicon probe
gold pins	Neuralynx	EIB Pins Small	
ground wire	A-M Systems	781500	0.010 inch bare silver wire
headstage preamp	Neuralynx	HS-36	
impedance meter	BAK electronics	Model IMP-2	1 kHz testing frequency
mineral oil	ZONA	36-105	for lubricating screws and wires
optical fiber	Doric	MFC_200/260-0.22_50mm_ZF1.25(G)_FLT	
Recording system	Neuralynx	Digital Lynx 4SX	
ruby fiber scribe	Thorlabs	S90R	for cleaving the optical fiber
silicon grease	Fine Science Tool	29051-45	
silicon probe	Neuronexus	A1x32-Edge-5mm-20-177	Fig. 3, 4A, 4B, 5
silicon probe	Neuronexus	A1x32-6mm-50-177	Fig. 4C
silicon probe washing solution	Alcon	AL10078844	contact lens cleaner
silicone lubber	Smooth-On	Dragon Skin 10 FAST	for preparation of microdrive mold
silver paint	GC electronic	22-023	silver print II coating, used for ground wires

skull screw	Otto Frei	2647-10AC	0.8 mm diameter, 0.200 mm thread pitch
standard surgical scissors	ROBOZ	RS-5880	
stereotaxic apparatus	Kopf	Model 942	
super glue	Loctite	LOC230992	for applying to guide-posts
surgical tweezers	ROBOZ	RS-5135	
Tetrode Twister	Jun Yamamoto	TT-01	
tetrode wires	Sandvik	PX000004	

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Title of Article:	Hybrid Microdrive System with Recoverable Opto-Silicon Probe and Tetrode for Dual-Site High Density Recording in Freely-Moving Mice
Author(s):	Hisayuki Osanai, Takashi Kitamura, and Jun Yamamoto

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Signature: Jun Yamamoto Date: 03/21/2019

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We are grateful to the two reviewers and the editor for the critical comments and useful suggestions that have helped us to improve our manuscript considerably. As indicated in the responses below, we have taken all these comments and suggestions into account in the revised version of our paper. In the revised manuscript, changes in the text are highlighted in red color except for the numbering of the Protocols and other minor corrections such as grammatical tenses and pluralities. Specific points changed in line with the reviewer's and editorial comments are as follows.

Editorial comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Thank you for giving us the opportunity to refine the manuscript. We have proofed our manuscript and corrected grammatical issues.

2. Authors and affiliations: Please provide an email address for each author.
We have added email addresses for remaining authors.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
We have checked and corrected the all numbering throughout the Protocol.

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This point has been corrected as following:
- **"Omenetics connector" to "silicon probe connector" in line 121, 376.**
- **"an Omnetics connecter of a 32-channel silicon-probe" to "a 32-channel silicon-probe connecter" in the legend of Fig. 1Cf line 457–548.**
- **"Omnetics" in line 310 is deleted.**
- **"a Dremel rotary grinder", "a Dremel grinder" to "a rotary grinder" in line 138, 155.**
- **"parafilm" to "plastic paraffin film" in line 377.**
- **"commercial photo-artifact reduced probes are available in NeuroNexus and Cambridge NeuroTech" to "there are photo-artifact reduced silicon-probes there are photo-artifact reduced silicon-probes commercially available" in Discussion in line 564-565**

5. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

This point has been corrected throughout the Protocol.

6. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

The sentences in the Protocol have been corrected to be described in the imperative tense in line 111–112, 238–239, 245, 301, 331–332, 343, 344–345, 357. In addition, we move the information of "NOTE" in the Protocol 5.14. to the Discussion line 561–562.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

This point has been corrected and refined as described below in 8–12. In addition, we added "using sharp scissors" in the Protocol line 175. We also added the instruction of using a hood during the perfusion of 4% paraformaldehyde in line 388.

8. Line 140: What is used to cut?

We have added "using a rotary grinder" in line 154.

9. Line 267: Please mention how proper anesthetization is confirmed.

We have added the sentence "The anesthetic condition of the mouse is confirmed by the absence of toe-pinch reflex." in line 322–323.

10. Line 270: How large is the incision? Please specify surgical tools used throughout the protocol.

We have described "a 1.5–2.0 cm incision" in line 327–328. We also mentioned the tools: "standard surgical scissors" in line 328, "using surgical tweezers and a screwdriver" in line 336, "using pliers" in line 399. The information of the surgical scissors and tweezers are also added in the Table of Materials.

11. Line 298: Please specify the size of the needle.

We replaced the words "a needle" to "a 20G needle" in line 362.

12. Line 318: Please describe how perfusion is done.

We have corrected the description in line 386–389 and added the reference as:

"Inject ketamine (75 mg/kg) and dexmedetomidine (1 mg/kg) anesthetics intraperitoneally and confirmed the absence of toe-pinch reflex. Fix the anesthetized mouse by directly perfusing 4% paraformaldehyde through the heart

using a hood. Surgical methods for rodents are described previously[42]"

Added reference:

42 Gage, G.J., Kipke, D.R., & Shain, W. Whole animal perfusion fixation for rodents. Journal of visualized experiments. (65), doi:10.3791/3564 (2012).

13. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Thank you for giving us the opportunity to refine the manuscript. This point has been corrected and refined in the Protocol 1.2.1., 1.2.2., 2.1.2., 2.1.3., 2.1.4., 2.2.1., 2.2.2., 2.4., 2.5., 2.7.3., 2.8.3., 2.8.4., 2.8.5., 2.8.7., 3.3., 3.4., 3.5., 3.6., 4.1., 4.3., 5.4., 5.6., 5.9., 5.10., 5.12., 5.13., 5.14., 6.2., 6.7.

14. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

We have corrected throughout the manuscript as the editor's instruction.

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted 2.5 pages of the Protocol after making all the recommended changes.

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

We have highlighted complete sentences in the Protocol. Notes are not highlighted.

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

All relevant details are included in the highlights.

18. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

We have separated each Figure and uploaded them.

19. Please upload Table 1 to your Editorial Manager account as an .xlsx file.

We have uploaded Table 1 and 2 as .xlsx files.

20. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

This point has been corrected.

21. References: Please do not abbreviate journal titles.
Thank you. This point has been corrected.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The paper describes a micro-drive to perform simultaneously tetrode recordings, silicon probe recordings and light stimulus delivery. The design of the micro-drive, the steps to build and implant are sufficiently described.

Minor Concerns:
line 78: maybe high cost instead of expensiveness
Thank you. We have replaced the word "expensiveness" in line 78 to "high cost".

line 106: Fig. 1A, B
Thank you. We have corrected this point in line 111.

line 133: 'anti-twist tube' is used, but on the figure 2B it is 'support tube'.
Thank you. We have replaced the word "an anti-twist tube" in line 147 to "a support tube".

It is not specified which parts of the drive need to be printed again and which parts are reused (do they need to be cleaned? How dental cement is removed?) for the next surgery.
Thank you very much for your suggestions. We have modified added two procedures in the recovery protocol in line 415–423:

> **"6.8. To reuse the microdrive array body for the next surgery, remove the dental acrylic using a combination of fine-tip drills and nippers. Then, recover the skull-screws by immersing the removed dental acrylic into acetone. Note that acetone will dissolve the plastic parts of the microdrive array.**
>
> **6.9 Remove the epoxy between the microdrive body and the shielding cone using a scalpel.**
>
> **Note: no additional parts are needed to be printed again for the next surgery if the microdrive is not broken."**

Reviewer #2:
Manuscript Summary:
I would like to thank the authors for their invention of a novel 3D printed microdrive design that allows for the simultaneous use of tetrode loaded microdrives and silicon probes alongside optic fibres. This new design of microdrive boasts to address a common

need in Neuroscience: multisite recordings from multiple regions with control of neuronal populations via light stimulation. This is an exciting advance in neuroscience and could be a innovative, cheap tool for many researchers.

Except for minor comments about the flow of the introduction (see Minor concerns), the manuscript reads very well and is clear and concise throughout. In particular, the abstract is informative, comprehensible and enjoyable to read. Alongside this, a thorough grasp of the motivations and difficulties facing researchers today is presented in the introduction alongside clear and logical reasoning behind the design of their microdrive. This level of discussion is very useful for scientists who use these tools.

The instructive steps laid out to build the microdrive are clear, easy to follow and logical. Instructions are complimented by informative and well structured figures throughout. All important points included in the instructions alongside useful tips and pointers that might achieve the best result. This level of detail is very much appreciated and will aid researchers achieve optimum results.

The representative results are very useful and provide a proof of concept to the microdrive design presented. The demonstration of the use of optogenetics to show interactions between distinct networks is particularly exciting.

The discussion of the drive design is clear, relevant and informative. The insider tips described to help reduce common problems with microdrives i.e. movement related noise, is very insightful and appreciated and shows a clear understanding of the necessities for successful recordings of this kind. Moreover, although weight of the drive is a concern (see Major concerns), I appreciate the weight is discussed and all relevant information is made available.

Overall the manuscript was very enjoyable and am very thankful for the authors contribution of this innovative, clever and highly useful microdrive design. If major concerns are met it could be a widely used tool in neuroscientific research. Moreover the microdrive design shows great dedication towards developing new tools to address complicated questions in science.

Major Concerns:
The only major concern held in regards to the manuscript is to the weight of the microdrive described and the presentation of the discussion around the weight of the drive and representative mouse implanted. My concerns, comments and questions in regards to this are listed below. Not all are major concerns but fall under the same topic so for clarity I have kept them together.

We thank Reviewer #2 for raising a critical point. The weight of the implanted device is important to be considered since it can prevent the animal's natural behaviors. It is often said the limit of the implant weight is ~10 % of the body weight, but actually there are no published data supporting this (Lidster, et al., 2006). In our microdrive case, as we described below, our implanted mice (>25 g) tolerated with the microdrive. Although the first few days during recovery needs attention, all the implanted mice showed standard behavioral performances such as walking, eating,

grooming and rearing and even jumping after the recovery period. These mice also performed behavioral tasks such as linear track running, so we consider our microdrive array does not restrict animal's behavior. Unfortunately, it is difficult to describe the limit of the mouse body weight since we have implanted only using the mice with >25 grams. However, because we have not seen issues in behavioral performances at least when we used these mice, we recommended the used of the mice larger than 25 grams.

1. Weight of the drive is primarily described in the discussion (lines 454 - 461) but weight of mice preferred for implantation is not mentioned (only that they be adults not juvenile). The weight is approximated in Figure 5 (line 250) where animal weight of over 25 g is noted. My first recommendation is that mouse weight and drive weight (as a percentage of mouse weight) should be included in the discussion paragraph for clarity.

Thank you very much for your suggestion. We have modified the Discussion as below to clarify the mouse weight and the drive weight (and the drive weight as a percentage of mouse weight):

In line 567–571,

"The weight of our microdrive array (5.9 grams) is heavier than the typical microdrives described in previous articles[12,53], mainly due to the microdrive array body (~21% of the total weight), shielding cone (~31%), and metal parts (screws and nuts; ~22%). We recommend to use the mouse with the weight of over 25 grams (~2–3 months old for C57BL/6 mice[54,55]) for implant surgery because the mice with the adequate body weight tend to recover earlier."

In line 572–575,

"While the devices of 5–10% of bodyweight are often guided to be tolerated for implant[12,56] although there are no supporting published data for this[55], our microdrive array weigh ~24% bodyweight of 25 grams mice (~19% when using the alternative cone described below). However, …"

Added references:

54 Hu, S. et al. Dietary Fat, but Not Protein or Carbohydrate, Regulates Energy Intake and Causes Adiposity in Mice. Cell Metabolism. 28 (3), 415-431 e414, doi:10.1016/j.cmet.2018.06.010 (2018).

55 Yang, Y., Smith, D.L., Jr., Keating, K.D., Allison, D.B., & Nagy, T.R. Variations in body weight, food intake and body composition after long-term high-fat diet feeding in C57BL/6J mice. Obesity. 22 (10), 2147-2155, doi:10.1002/oby.20811 (2014).

55 Morton, D.B. et al. Refinements in telemetry procedures. Seventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement, Part A. Laboratory Animals. 37 (4), 261-299, doi:10.1258/002367703322389861 (2003).

56 Lidster, K. et al. Opportunities for improving animal welfare in rodent

models of epilepsy and seizures. Journal of neuroscience methods. 260, 2-25, doi:10.1016/j.jneumeth.2015.09.007 (2016).

Also, we have clarified the reason of the use of >25 grams mice in the NOTE of the Protocol line 301–302 as "…for the faster recovery after the surgery.".

2. My second concern is that if mice that are 25 g are implanted and your drives are approximately 5.9 g this means your drive is approximately 24 % of your mouses body weight. But since only a vague weight is given ("over 25 g"), I am unsure of the exact weight of mice implanted that was successful and resulted in the "jumping" behaviour in the home cage you observed and describe in lines 457 - 458. Can you be more explicit about exact weight of the animal here alongside the age and gender.

Thank you very much for your suggestion. We have added the description of the mouse in the Representative Results line 428–429:

"21 weeks old, 29 g body weight male pOxr1-Cre mouse (C57BL/6 background)"

3. If the actual weight of mouse to drive is as described (approx. 24%), some researchers may struggle to use such the device for reasons described in Voigts et al., 2013 (also referenced in the manuscript). Moreover, in some countries Veterinary law restricts weights of devices implanted into animals. For this reason, could you add more detail on how the drive design could be modified to make it lighter? Specially you discuss reducing height and thickness of the cone. Could you give the range of heights and thicknesses you advise, and comment on the effect changing these parameters might have (i.e. drive stability).

Thank you very much for your suggestion. Since the shielding cone is the heaviest part of our microdrive array, the modification in the cone can easily reduce the total weight. However, thinning the cone in excessive way (less than 0.3 mm) make the cone soft and easy to break. In addition, excessive lowering the cone height (more than ~ 5 mm) make the tetrode wires exposed and cause their break. If the researchers do not require physically strong protection but only the electromagnetic shielding, it is possible to prepare the shielding cone using a paper and aluminum tape which greatly reduce the total implant weight (reduce ~20% of the total weight). Thus, we added the following sentence in the Discussions:

In the Discussion line 582–588,

"Using the current 3D-printing material, the thickness of the shielding cone can be reduced up to ~0.3 mm from the current thickness of ~0.6 mm. The cone height can be reduced ~5 mm as long as the tetrode wires can still be covered; exposure of the tetrode wires will result in breakage of the wires and a failure of the long-term recording. Alternatively, preparation of the shielding cone using a paper and aluminum tape can reduce the cone weight to ~0.7 grams (~15% of total weight; reduce 20% from the total weight of the original microdrive array), although these are a tradeoff with the physical strength."

We have also added the ratio of the body/cone/metal parts to the total weight of the original microdrive array in line 568-569 in order to help readers to compare these weights.

In addition, we have added Figure 3F and following sentences in the Protocol to explain how to prepare the alternative shielding cone using a paper and aluminum foil. The total weight in the case of using this cone have been described in Table 1.

In the Protocol line 292–296,

"Note: Another way to prepare the shielding cone is to use aluminum tape40 (Fig. 3F). First, prepare the pattern paper for the shielding cone after sticking the alumni foil to the paper (Fig. 3Fa). Then, roll paper and attach to the microdrive body using a small amount of cyanoacrylate glue (Fig. 3Fb). The weight of this cone is 0.72 grams and the total weight of the microdrive array is reduced to 4.7 grams (Table 1)."

In the Figure 3 legend line 486–488,

"(G) Alternative shielding cone using a paper and aluminum tape. (a) A pattern paper. (b) An attached alternative shielding cone, which reduces 1.1 grams of weight compared to the 3D printed version."

In the Table 1 legend line 527–528, we have also added:

"*: in the case of using an alternative shielding cone using a paper and aluminum tape."

The information of the aluminum foil tape has been added in the Table of Materials.

4. Could you also include in the discussion the total dimensions of the protective cone and a comment on whether it prevents usual mouse behaviour in the home cage due to size i.e. feeding, cleaning and grooming.

Thank you very much for your suggestion. We have added the size of the protective cone as following. The implant on the mouse head can be an obstacle to foods or water access which provided from the cage top. However, as long as they are provided on the floor or from the sidewall, we do not observe any issues in mouse behaviors such as eating, drinking, grooming, rearing, or nesting.

Thus, we added the following sentence in the Discussions:

In line 588–593,

"In addition, the size of the microdrive (current shielding cone: 4.2×4.0×2.6 cm in major axis×minor axis×height) can be an obstacle to food and water access if they are provided from the top of the animal cage. As long as they are provided on the cage floor of from the sidewall, the microdrive does not disturb natural behaviors of mice, such as eating, drinking, grooming, rearing, or nesting[60]."

Added references:

60 Gaskill, B.N., Karas, A.Z., Garner, J.P., & Pritchett-Corning, K.R. Nest building as an indicator of health and welfare in laboratory mice. Journal of visualized experiments. (82), 51012, doi:10.3791/51012 (2013).

In addition, we added the implanted mouse photos during walking and grooming in Figure 4 as described below in 5.

5. Could a photo of a mouse be included that shows the mouse with implant after the recovery period. This may be appreciated as researchers can visualise the impact of the implant size and make a more informed decision as to whether it is appropriate for their research purposes.

Thank you for your suggestion. We have added the photos of the implanted mouse after the recovery period in Figure 4Ca-c. We also added the following sentences to describe these photos:

In the Protocols line 382,

"The implanted mouse after the recovery period is shown in Fig. 4C."

In the legend of figure 4 line 493–495,

"(C) The chronically implanted mouse after the recovery period, when the mouse is walking (a), grooming (b), and when connected to the recording cable with the counter-balancing pulley system (c)."

Minor Concerns:

1. Use of the word "probe" (line 55) to refer to all recording devices may be confusing as tetrode microdrives are not usually referred to as probes but are one of the categories discussed under "probes". Perhaps use "recording devices" or "electrodes" i.e. tetrodes, silicon probes for clarity.

Thank you. We have replaced the word "probes" to "electrodes" in line 57.

In addition, we modified the sentence in the Introduction line 90–91, "to optimize the selection of the type of electrode/probes" into "to optimize the selection of the type of electrode or to implant an optical fiber"

2. The introduction contains clear and concise motivation behind the design drive but it does not read as well as it could. The flow is interrupted as paragraphs are split inappropriately and there isn't sufficient linking and summarising statements between main points. Can you ensure each paragraph is self contained (start with a sentence introducing your point and end on a summary sentence at the end). Specific examples where this is problematic in the introduction:

a. Paragraphs covering lines 60-78 : the two paragraphs are arguing towards the same point or conclusion. It might flow better if paragraphs are joined or made self contained i.e change the start of the second paragraph (starting at line 69) from "On the other hand" to something like "In contrast to tetrodes, silicon probes…".

b. Similar issue as above at paragraphs 80-87

c. At line 90 could start a new self contained paragraph that summarises points in your introduction.

Thank you very much for your suggestions. We have refined the Introduction as following:

a. In line 61-80, we combined the two paragraphs about tetrodes and silicon probes,

and added the following summary sentence in the last part of this paragraph.

In line 78–80,
"Thus, the choice of the tetrodes or the silicon probes depends on the aim of the recording: whether the researchers prioritize to obtain the high yield of single-unit or spatial profile at the recording sites"

b. In line 87-88, we added the summary sentence of the paragraph:

"Therefore, it is not trivial task to combine the implantable optical fiber to the conventional microdrive arrays."

c. We separated the reviewer's pointed sentences and combined with the last paragraph of the Introduction (line 90-104). Accordingly, we refined the sentences in this paragraph line 99–100

3. Where three levels of subheadings are used in the instructions, the third level is a bit unclear. I.e. those denoted with 1.1. Could you indent them once more? This might not be necessary and could be my personal preference.
Thank you for your suggestion. We have indented at the third level of the subheadings through the Protocol.

4. At line 267 the numbering jumps from 1. To 4. (2 and 3 are missed out).
Thank you. We have corrected the numbering throughout the Protocol.

5. At line 267, can you comment on whether ointment is clear or opaque that you apply to the eyes of animals?
Thank you for your suggestion. We have added "Apply clear ointment" in the Protocol line 323. In addition, to describe that the mice are protected from surgical light during surgery, we added the following sentence in line 324–325: "Cover the eyes with a piece of foil to protect from strong surgical light exposure."

We have also added the information of the ointment in the Table of Materials.

6. Time spent to build is often an important consideration for scientists, could you give an approximate time to make the drive by stage? i.e. include a brief timeline/table of how long each drive building step takes. Does not need to be detailed but more of a summary overview of main steps.
Thank you for your suggestion. We added Table 2 for the time line of the microdrive preparation (in total within 4–5 days). To describe about Table 2, we have added the following sentences in the manuscript:

In the Representative Results line 426–427,
"The microdrive array was constructed within 5 days. The timeline of the microdrive preparation is described in Table 2. Using this microdrive, …"

In the legend of Table 2 line 530–532,

"Table 2. The timeline of the microdrive preparation. The 3D-parts printing, waiting for curing the silicone rubber/dental acrylic/epoxy, and loading the tetrode wires take the majority of the time of the microdrive array preparation, in total 4–5 days."

7. Is it possible to show spikes obtained from the MEC in a similar way to how its shown for the CA1? If not, can you discuss why this is not included in the representative results? Or if I am misunderstanding the figure can you make it clearer where this is shown?
Thank you for your suggestion. We have added the representative result of the MEC spikes in Figure 6C which is referred in the Representative Results line 443. We also added the following sentences:

In the legend of Figure 6 line 516–520,

"(C) Representative spike recording from one of the silicon-probe electrode sites. (a) Two-dimensional cluster projections of spike principal components. (b) Examples of the average spike waveform of three clusters. Spike clusters (pink and green) are separated from the noise clusters (blue). The clusters in (B, C) are calculated using KlustaKwik software."

Accordingly, we also modified line 521–522 as:

"Black arrows indicate the tetrode shown in (B) and the silicon-probe electrode site shown in (C)."

8. Could you comment under "representative results" whether data shown was gathered in anesthetised or awake animals? If awake, could you describe the behavioural state of the animal during the representative recording, was it still or moving at the time of the recording?
Thank you for pointing the critical comment. We have recorded electrophysiological data from the naturally sleeping mouse without anesthetics. Thus, we added the word "when the mouse was sleeping" in the Representative Results line 444.

In addition, in order to demonstrate that our microdrive is able to achieve the electrophysiological recording from behaving animals, we additionally added Figure 5 (original Figure 5 was moved to Figure 6 in the current version), which represents the results of the recording on the linear track and in the open field.

In the Representative Results line 438–442, we have added

"After the tetrode adjustment, behavioral performance was tested on a linear track (Fig. 5A) and in an open field (Fig. 5B). In both experiments, the mouse explored freely for ~30 min (Fig. 5Aa-c, Ba-c). The electrophysiological signals were successfully recorded without severe motion-related noise throughout the recording session (Fig. 5Ad, Ae, Bd, Be). Next, light stimulation was performed at the MEC to stimulate MEC layer III neurons which project to the CA1[43]…"

We have also added the legend of Figure 5 in line 499–508:

"Figure 5. Examples of the simultaneous tetrode/silicon-probe recording in the hippocampal CA1 and the medial entorhinal cortex (MEC) from the behaving

mouse. (A) Recording on the linear track. (a) The linear track used for the recoding. (b) Trajectories of the mouse exploration for ~30 min on the track. (c) Behavioral performance on the linear track. (d-e) Representative LFP recordings from the tetrode (d) and the silicon-probe (e). (B) Recording in the open field. (a) The open field chamber used for the recoding. (b) Trajectories of the mouse exploration for ~30 min in the chamber. (c) Behavioral performance in the open field. (d-e) Representative LFP recordings from the tetrode (d) and the silicon-probe (e). LED is attached to the head amplifier to record the positions of the mouse. The linear track and the open-field chamber are connected with the electrical ground to reduce electrostatic noise."

9. Aversive stimuli is often used in behavioural experiments to motive the animal i.e. water and food restriction. Do you think the recommended weight of the animal should be reconsidered for behavioural experiments that require food and water restriction? If so could you comment on this in the discussion?

Thank you for your critical comment. We have already successfully conducted T-maze task using the food-restricted mice implanted with the microdrive (4.5 grams) similar to the current manuscript. In our experience, the adequate mouse weight is needed for the fast recovery after the surgery. However, once the mice get used to carry the implanted device, the mice do not show any problems in their behavioral performances even when they are food deprived, probably because the mice have acquired and strengthened the muscles to support the implant on their head.

We have added the following sentence about the previous food-restricted mice experiments:

In Discussion line 576–578,

"The mice implanted with the similar microdrive array weight (~4.5 grams) have previously been shown to perform the behavioral task (linear maze task) even under the food restriction[13,17]."

10. The table is very clear and all components listed are easy to understand. One comment that is perhaps my own misunderstanding - under "customized screws for tetrode microdrive" is the "Yamamoto_0000- 160_9mm " a quote they have sent you or design you have sent in for the custom screw? If you think it's necessary a comment here could be useful to clarify.

Thank you for pointing this. The screws are custom-made to accommodate for our design. In the Table of Material, we have added "custom-made to order for our design".

11. In figure 5 legend, could you comment on the software used to do the spike sorting described in (B) (a).

Thank you for your suggestion. We have added the sentence in figure 6 legend line 519–520: "The clusters in (B, C) are calculated using KlustaKwik software.".

Other alterations made voluntarily:

Because we had found four minor errors in our manuscripts during the revision, we have corrected the following errors:

1. We have corrected the following texts regarding to the isoflurane concentration in line 322, “0.8–3.0% isoflurane” to “0.8–1.5% isoflurane”.

2. We have added a reference regarding to the projection from MEC layer III to CA1 in line 430:

   43 Suh, J., Rivest, A.J., Nakashiba, T., Tominaga, T., & Tonegawa, S. Entorhinal cortex layer III input to the hippocampus is crucial for temporal association memory. Science. 334 (6061), 1415-1420, doi:10.1126/science.1210125 (2011).

3. We have slightly modified the Fig.1Ab (the position of the microdrive screw and wires).

4. To increase the visibility, we have added the labels in in Fig. 3Ca, b.