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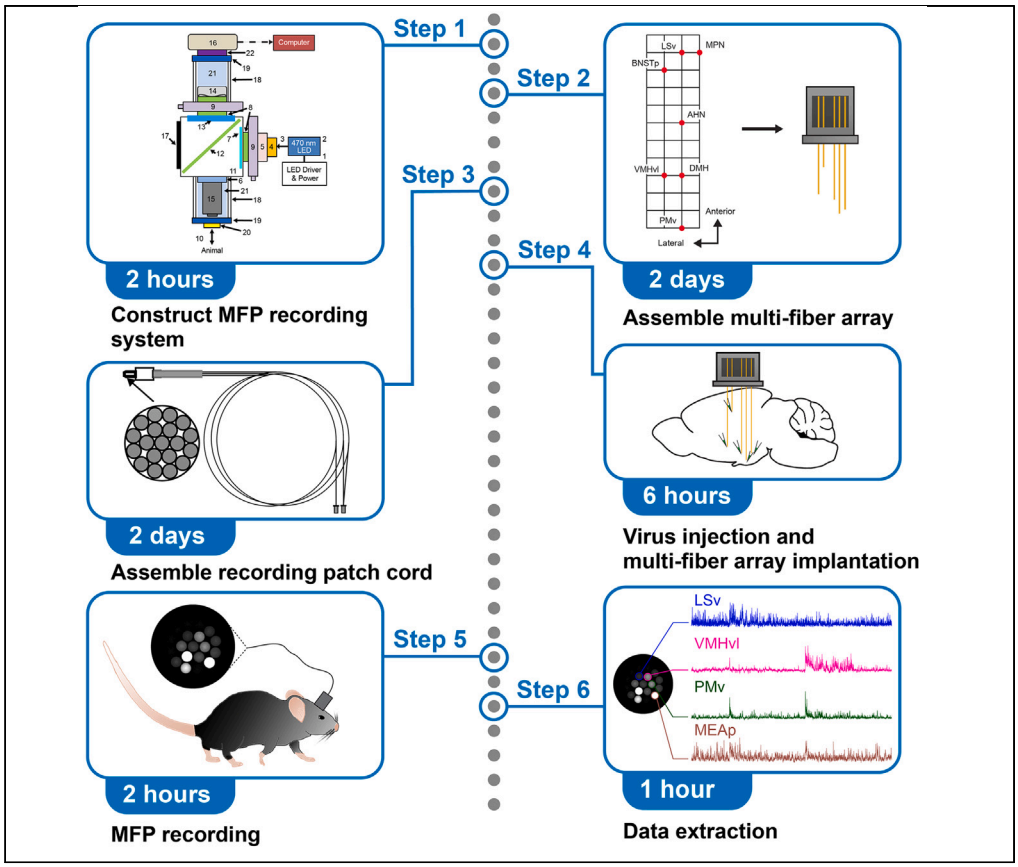
Brain-wide multi-fiber recording of neuronal activity in freely moving mice



While brain regions function in coordination to mediate diverse behaviors, techniques allowing simultaneous monitoring of many deep brain regions remain limited. Here, we present a multi-fiber recording protocol that enables simultaneous recording of fluorescence signals from multiple brain regions in freely behaving mice. We describe steps for assembling a multi-fiber array and patch cord, implantation, and recording. We then detail procedures for data extraction and visualization. This protocol enables a comprehensive view of the neural activity at the network level.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Bing Dai, Zhichao Guo, Dayu Lin

bd1409@nyu.edu (B.D.)
dayu.lin@nyulangone.org (D.L.)

Highlights

Detailed instructions for constructing a cost-effective multi-fiber photometry system

Steps described for constructing custom multi-fiber arrays

Guidance on array implantation, *in vivo* recording, and data extraction

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Brain-wide multi-fiber recording of neuronal activity in freely moving mice

Bing Dai,[1,4,*] Zhichao Guo,[1,3] and Dayu Lin[1,2,5,*]

[1]Neuroscience Institute, New York University Langone Health, New York, NY 10016, USA

[2]Department of Psychiatry, New York University Langone Health, New York, NY, USA

[3]School of Life Sciences, Peking University, Beijing 100871, China

[4]Technical contact

[5]Lead contact

*Correspondence: bd1409@nyu.edu (B.D.), dayu.lin@nyulangone.org (D.L.)
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SUMMARY

While brain regions function in coordination to mediate diverse behaviors, techniques allowing simultaneous monitoring of many deep brain regions remain limited. Here, we present a multi-fiber recording protocol that enables simultaneous recording of fluorescence signals from multiple brain regions in freely behaving mice. We describe steps for assembling a multi-fiber array and patch cord, implantation, and recording. We then detail procedures for data extraction and visualization. This protocol enables a comprehensive view of the neural activity at the network level.

For complete details on the use and execution of this protocol, please refer to Guo et al.[1]

BEFORE YOU BEGIN

Fiber photometry is a method for recording fluorescence signals from a population of cells using optic fibers implanted in the brain.[2,3] This method was initially developed to record a single brain site and later expanded to simultaneously record from multiple brain regions, namely multi-fiber photometry (MFP).[4] MFP is proven valuable for monitoring neural network dynamics and has been employed in many neuroscience studies. With its increased popularity, commercial systems are now available to achieve simultaneous recordings from up to 7 sites (e.g., Doric lens). However, commercial systems are costly and have limited channel capacity. In 2019, Sych et al.[5] expanded the original multi-fiber photometry system to achieve simultaneous recording of 48 brain sites in freely moving mice. We adopted the system introduced by Sych et al. to record from 13 regions in the limbic system during sexual and aggressive behaviors in male mice.[1] In the process of adopting the system, we simplified the setup, optimized the fiber construction and implantation procedure, and developed custom MATLAB code to extract the recording signals. These improvements made this method more affordable and user-friendly. The current protocol focuses on our procedures of multi-fiber array assembly, surgical implantation, and recording. While we have chosen mice as our animal model, these methods can be readily applied to other small animals. Additionally, although this protocol focuses on recording calcium signals, we have successfully applied the method to record dopamine signals using $GRAB_{DA}$ sensors,[6] demonstrating the versatility of the approach in studying dynamics of neurochemical signals.

Institutional permissions

All procedures were approved by the IACUC of NYULH in compliance with the NIH guidelines for the care and use of laboratory animals. The users of the protocol must acquire similar permissions from their relevant institutions.

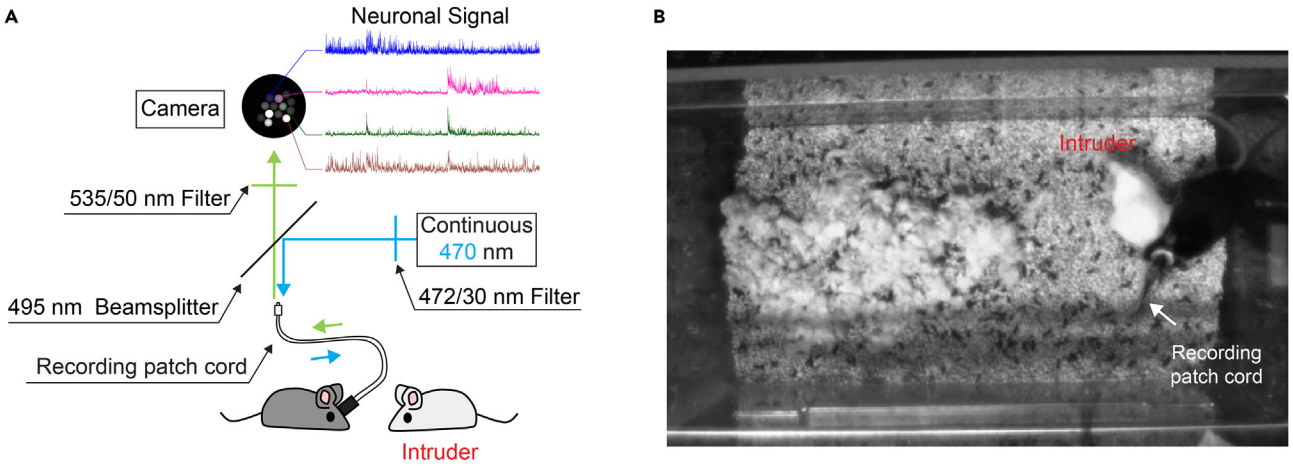


Figure 1. Overview of the multi-fiber photometry recording system and an example frame during recording

(A) The system utilizes a 470-nm LED as a constant light source. The light initially passes through a 472/30-nm single-band bandpass filter, which narrows the excitation light's wavelength. This excitation light is then reflected by a 495-nm dichroic beamsplitter, designed to reflect light wavelengths below 495 nm while allowing those above to pass. After reflecting off the beamsplitter, the excitation light travels through the recording patch cord, illuminating neuronal sensors like GCaMP6. The emission light produced by these sensors again traverses the recording patch cord, passes through the beamsplitter, and is subsequently filtered by a 535/50-nm single-band bandpass filter. Finally, this filtered light is captured by the camera. The signals recorded are then extracted and analyzed using MATLAB.

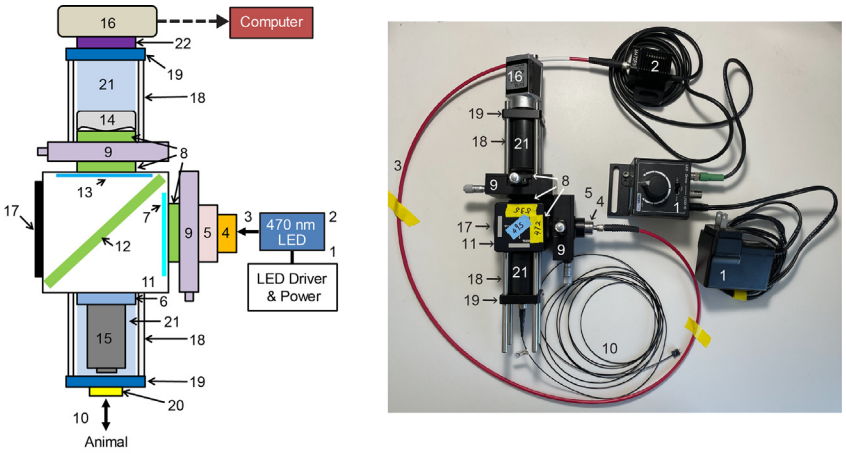
(B) An example frame from an MFP recording session shows the test mouse freely interacting with the intruder.

Set up the multi-fiber recording system

Timing: 2 h

As illustrated in Figure 1, the system utilizes a 470-nm LED as a constant light source. The light initially passes through a 472/30-nm single-band bandpass filter, which narrows the excitation light's wavelength. This excitation light is then reflected by a 495-nm dichroic beamsplitter, designed to reflect light wavelengths below 495 nm while allowing those above to pass. After reflecting off the beamsplitter, the excitation light travels through the recording patch cord, illuminating neuronal sensors like GCaMP6. The emission light produced by these sensors again traverses the recording patch cord, passes through the beamsplitter, and is subsequently filtered by a 535/50-nm single-band bandpass filter. Finally, this filtered light is captured by the camera. The signals recorded are then extracted and analyzed using MATLAB.

1. Prepare all the components as illustrated in Figure 2.
2. Connect the T-Cube LED driver (LEDD1B, Thorlabs) to the T-Cube power supply (KPS201, Thorlabs) and the 470 nm LED (M470F4, Thorlabs), as shown in Figures 3A and 3B.
3. Attach the SMA-SMA fiber patch cable (M59L01, Thorlabs) to the 470 nm LED (Figure 3A).
4. Insert the multimode collimator (F950SMA-A, Thorlabs) into the SM1-threaded adapter (AD15F, Thorlabs) and secure it with a hex key (Figures 3C and 3D).
5. Attach the adapter and collimator to an XY translator (ST1XY-S, Thorlabs) (Figure 3E).
6. Remove the insert of the kinematic fluorescence filter cube (DFM1, Thorlabs) and disassemble it using a hex key (Figures 3F and 3G).
7. Place the 495 nm dichroic beamsplitter (FF495-Di03-25 × 36, Semrock) into the disassembled filter cube (Figure 3G) and reassemble the filter cube.
8. Use a spanner wrench (SPW602, Thorlabs) to remove the retaining rings from the filter cube (Figure 3H).
9. Install both the 472/30 nm and 535/50 nm single-band bandpass filters (FF02-472/30-25 and FF01-535/50, Semrock) onto the filter cube and secure them with retaining rings (Figures 3H and 3I).



No.	Name	SOURCE	IDENTIFIER	QUANYITY
1	T-Cube Power Supply	Thorlabs	KPS201	1
	T-Cube LED Driver	Thorlabs	LEDD1B	1
2	470 nm LED	Thorlabs	M470F4	1
3	SMA-SMA Fiber Patch Cable	Thorlabs	M59L01	1
4	SMA905 Multimode Collimator	Thorlabs	F950SMA-A	1
5	SM1-Threaded Adapter	Thorlabs	AD15F	1
6	SM1 to RMS Adaptor	Thorlabs	SM1A3	1
7	472/30 nm Single-band Bandpass Filter	Semrock	FF02-472/30-25	1
8	SM1 coupler	Thorlabs	SM1T2	3
9	XY Translator with Micrometer Drives	Thorlabs	ST1XY-S	2
10	Custom-made 100μm Fiber Bundles	Doric Lenses	N/A	1
11	Kinematic Fluorescence Filter Cube	Thorlabs	DFM1	1
12	495 nm Dichroic Beamsplitter	Semrock	FF495-Di03-25x36	1
13	535/50 nm Single-band Bandpass Filter	Semrock	FF01-535/50	1
14	Achromatic Doublet	Thorlabs	AC254-060-A-ML	1
15	Olympus PLN 10X Objective	Edmund Optics	1-U2B223	1
16	Basler ace acA640-120um camera	Basler	acA640-120um	1
17	SM1-Threaded End Cap	Thorlabs	SM1CP2M	1
18	Cage Assembly Rod	Thorlabs	ER4-P4	2
19	SM1-Threaded 30 mm Cage Plate	Thorlabs	CP33	2
20	SMA Fiber Adapter Plate	Thorlabs	SM1SMA	1
21	SM1 Slip-On Lens Tube Cover	Thorlabs	SC1L24	1
22	Adapter with External C-Mount Threads and External SM1 Threads	Thorlabs	SM1A39	1

Figure 2. Schematic illustration and picture of the multi-fiber photometry recording system (top) and a list of all the components (bottom)

Couple the insert and base of the filter cube and attach the end cap (SM1CP2M, Thorlabs) (Figures 3I and 3J).

⚠ CRITICAL: The dichroic beamsplitter and bandpass filters are delicate components and must be handled with utmost care, preferably in a clean environment. Users should avoid touching the filter surfaces to prevent contamination.

10. Connect the Olympus PLN 10X objective lens (1-U2B223, Edmund Optics) to the filter cube using an SM1 to RMS adapter (SM1A3, Thorlabs) (Figures 3K and 3L).
11. Mount the SMA fiber adapter plate (SM1SMA, Thorlabs) onto an SM1-threaded 30 mm cage plate (CP33, Thorlabs) (Figures 3M and 3O).
12. Insert four cage assembly rods (ER4-P4, Thorlabs) into the 30 mm cage plate (Figure 3P).
13. Cut the 24 inches slip-on lens tube cover (SC1L24, Thorlabs) into two short pieces: one is 4 cm, and the other is 5 cm.
14. Cover the 10× objective lens with a 4 cm slip-on lens tube cover and connect the filter cube to the cage plate (Figures 3P and 3Q).
15. Replace the original lens of the camera (e.g., acA640-120 μm, Basler) with an adapter featuring external c-mount threads and external SM1 threads (SM1A39, Thorlabs). Then, connect the adapter to an SM1-threaded 30 mm cage plate (Figures 4A–4D).

Figure 3. Step-by-step assembly of the multi-fiber photometry recording system (part I)

(A and B) Connect the power supply, LED driver, LED, and SMA-SMA patch cord.

(C–E) Attach the multimode collimator to an XY translator.

(F–J) Install the fluorescence filters into the fluorescence filter cube.

(K and L) Attach the 10x objective to the fluorescence filter cube.

(M–Q) Attach the SMA fiber adapter plate to the fluorescence filter cube.

Note: Cameras equipped with adjustable frame rate (FPS $\geq$ 25) and exposure time settings are suitable for this purpose.

16. Mount the achromatic doublet (AC254-060-A-ML, Thorlabs) on the XY translator using an SM1 coupler (SM1T2, Thorlabs) (Figures 4E–4G).
17. Add an additional SM1 coupler to the other side of the XY translator (Figure 4G).
18. Install four cage assembly robs on the XY translator and cover the achromatic doublet with a 5 cm slip-on lens tube cover (Figure 4H).
19. Assemble the XY translator with the camera, as illustrated in Figures 4H and 4I.
20. Connect two XY translators with the filter cube via SM1 couplers (Figures 4J–4M).
21. Attach the SMA-SMA fiber patch cable to the multimode collimator (Figure 4N).
22. Power up the Basler camera and connect it to the computer using a Gigabit PoE Injector (TL-PoE150S, TP-LINK).

Figure 4. Step-by-step assembly of the multi-fiber photometry recording system (part II)
(A–D) Connect the Basler camera with a 30 mm cage plate.
(E–I) Attach the camera to the XY translator.
(J–M) Connect two XY translators with the fluorescence filter cube.
(N) Attach the SMA-SMA fiber patch cable to the multimode collimator.

23. Install StreamPix (StreamPix 8, NorPix) or any video recording software for camera operation and recording.

Note: Ideal recording software should have the capability to simultaneously manage at least three cameras, offer options to adjust the exposure time and frame rate for each camera, and be able to generate time stamps for every image captured.

24. The multi-fiber recording system is now ready.

3D print the head-fixation ring and apparatus

Timing: 8 h

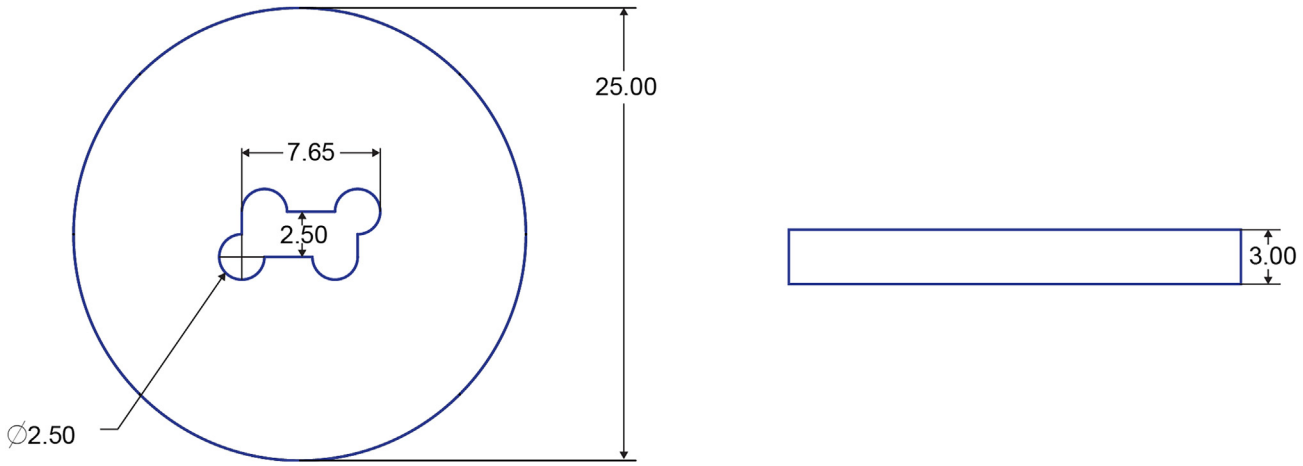


Figure 5. Dimensions of the polishing disk (unit: mm)

25. This head-fixed apparatus allows us to secure the head of the test mouse for connecting and disconnecting the patch cord without anesthesia. It was designed by Dmitry Rinberg Lab, and you can download the files from https://www.rinberglab.com/files. Same files can also be found in the Supplementary materials.

Note: All the supplementary materials are available at https://zenodo.org/doi/10.5281/zenodo.8428784.

26. Print each component using a 3D printer. We used MAKERGEAR M2 3D Printer and 1.75 mm PLA Filament (3D Solutech).
27. Follow the instructions in 'readme.txt' to assemble the head-fixation system.

3D print fiber-polishing disks

Timing: 5 h

28. Figure 5 shows the design of the polishing disk. It is used to polish the ends of fiber arrays. It was published originally in ref. 5 CAD file can be requested from Dr. Yaroslav Sych.
29. Print the polishing disk using a 3D printer. We used Formlabs Form 2 SLA 3D printer and clear resin (RS-F2-GPCL-04, Formlabs).

Install MATLAB and required toolboxes

Timing: 30 min

30. Download MATLAB 2023a from https://www.mathworks.com/products/matlab.html. A license is required.
31. Install MATLAB Image Processing toolbox and MATLAB Bioinformatics toolbox.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
AngstromBond room temperature cure epoxy	Fiber Optic Center	Cat#AB9112-2.5G
Ethanol 200 proof	Decon Labs, Inc.	Cat#2701, CAS#64-17-S

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Esr1-Cre male mice (10–24 weeks)	Jackson Laboratory	JAX: 017911
Deposited data		
MFP extraction code	This study	https://zenodo.org/doi/10.5281/zenodo.8428784
Recombinant DNA		
AAV1-CAG-Flex-GCaMP6f-WPRE.SV40	Addgene	Addgene: 100835-AAV1
Software and algorithms		
MATLAB R2023a	MathWorks	https://www.mathworks.com
StreamPix 8	NorPix	https://www.norpix.com/products/streampix/streampix.php
Other		
T-Cube power supply	Thorlabs	Cat#KPS201
T-Cube LED driver	Thorlabs	Cat#LEDD1B
Fiber-coupled 470 nm LED	Thorlabs	Cat#M470F4
SMA-SMA fiber patch cable	Thorlabs	Cat#M59L01
SMA905 multimode collimator	Thorlabs	Cat#F950SMA-A
SM1-threaded adapter	Thorlabs	Cat#AD15F
SM1 to RMS adapter	Thorlabs	Cat#SM1A3
472/30 nm single-band bandpass filter	Semrock	Cat#FF02-472/30-25
SM1 coupler	Thorlabs	Cat#SM1T2
XY translator with micrometer drives	Thorlabs	Cat#ST1XY-S
Custom-made 100 μm fiber bundles	Doric Lenses	NA
Kinematic fluorescence filter cube	Thorlabs	Cat#DFM1
495 nm dichroic beamsplitter	Semrock	Cat#FF495-Di03-25 × 36
535/50 nm single-band bandpass filter	Semrock	Cat#FF01-535/50
Achromatic doublet	Thorlabs	Cat#AC254-060-A-ML
Olympus PLN 10X objective	Edmund Optics	Cat#1-U2B223
Basler ace acA640-120um camera	Basler	Cat#acA640-120um
SM1-threaded end cap	Thorlabs	Cat#SM1CP2M
Cage assembly rod	Thorlabs	Cat#ER4-P4
SM1-threaded 30 mm cage plate	Thorlabs	Cat#CP33
SMA fiber adapter plate	Thorlabs	Cat#SM1SMA
SM1 slip-on lens tube cover	Thorlabs	Cat#SC1L24
Adapter with external C-mount threads and external SM1 threads	Thorlabs	Cat#SM1A39
Spanner wrench	Thorlabs	Cat#SPW602
TP-LINK 802.3af Gigabit PoE injector	TP-Link	Cat#TL-PoE150S
MT ferrule with boot, 48 F	US Conec	Cat#12599
Guide pin, multimode, flat head	US Conec	Cat#12766
FC-6S high precision optical fiber cleaver	KELUSHI, Amazon	Cat#FC-6S
Polishing sheets, 5 μm grit	Thorlabs	Cat#LF5P
Polishing sheets, 1 μm grit	Thorlabs	Cat#LF1P
Polishing sheets, 0.3 μm grit	Thorlabs	Cat#LF03P
Standard photodiode power sensor, Si, 400–1,100 nm, 50 nW–50 mW	Thorlabs	Cat#S120C
Compact power and energy meter console	Thorlabs	Cat# PM100D
3M Wetordry sandpaper, P400	3M, Amazon	Cat#213Q
ZEISS Stemi 2000 stereo microscope with table stand	ZEISS	Cat#SP-STEMI2000-TS2
Kopf stereotaxic system	Kopf Instruments	Cat#Model 1900
Fiber optic cannulae with ceramic ferrule	RWD Life Science	Cat#R-FOC-L200C-50NA
Microinjector	WPI	Cat#Nanoliter 2020
100 μm core multimode fiber	Thorlabs	Cat#UM22-100
BD 1 mL tuberculin syringes	Fisher Scientific	REF#BD30659

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BD PrecisionGlide needles, 22G	Fisher Scientific	REF#BD305155
Kimwipes delicate task wipers	Fisher Scientific	Cat#06-666A
Drill bits #79 (0.0145)	Drill Bit City	Cat#10WS79-550FL
Flow-IT ALC flowable dental composite	Henry Schein	Cat# N11H
Falcon Dust-Off compressed gas cleaning duster	Amazon	Cat# 8541677579
3D printer premium PLA filament 1.75 mm filament	3D Solutech, Amazon	Cat#2012026
Clear resin	Formlabs	Cat# RS-F2-GPCL-04
Hartman hemostats	Fine Science Tools	Cat# 13003-10
Duster		
C&B Metabond quick adhesive cement system	Parkell	Cat# S380
Krazy Glue Elmer's original Crazy Super glue	Krazy Glue, Amazon	Model# KG585

STEP-BY-STEP METHOD DETAILS

Assemble multi-fiber array

Timing: 2 days

This section describes the preparation of the multi-fiber array.

1. Design the multi-fiber array.
   a. The MT ferrules used for constructing multi-fiber arrays are purchased from US Conec.

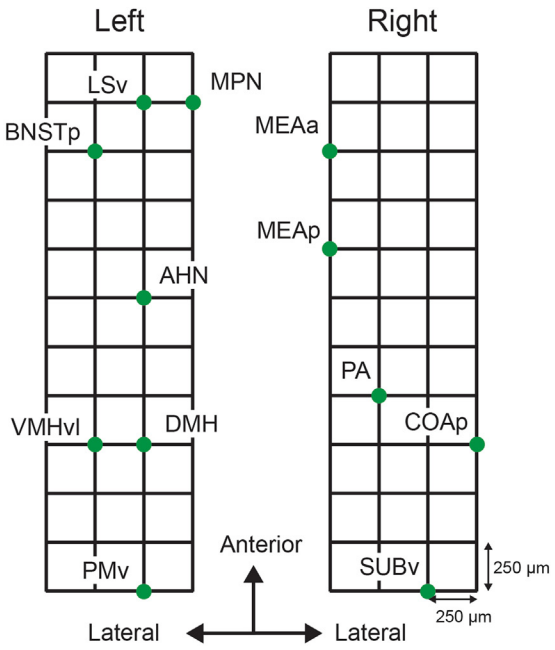
   Note: The company offers several different ferrule configurations, which can be viewed at: https://www.usconec.com/umbraco/rhythm/protectedfilesapi/download?path=%2ffiles%2fdrawings%2fC7593.pdf. The dimensions for each ferrule type are also available on the company website. For example, the drawing of the 48-channel ferrule (MT Ferrule with boot 48 F, Cat#12599, US Conec) can be found here: https://www.usconec.com/umbraco/rhythm/protectedfilesapi/download?path=%2ffiles%2fdrawings%2fC12405.pdf. (These two pdf files can be found in the Supplementary materials)

   b. Determine the channels to use and the length for each fiber based on the regions of interest (ROIs) and the dimension of the selected ferrule.

   Note: In our example, we have chosen 12 specific brain regions for multi-fiber recording. Figure 6 shows the Bregma coordinates for each recording region and the corresponding channel chosen in the MT ferrule.

   ⚠ CRITICAL: MT ferrules have a fixed center-to-center spacing of 250 μm between channels and may not allow targeting all the ROIs precisely. You have the option to customize a ferrule and produce it using a high-resolution 3D printer. See problem 1.

2. Fiber preparation and insertion.
   a. Prepare necessary materials (Figure 7A). Adjust the blade of the fiber cleaver (FC-6S, KELUSHI) to the appropriate position for cutting 100 μm fiber (Figure 7B).
   b. Use ethanol and low-lint wipers (e.g., Kimwipes delicate task wipers, Fisher Scientific) to clean the 100 μm fiber (UM22-100, Thorlabs).
   c. Load the 100 μm fiber onto the fiber cleaver.
   d. Use a ruler to measure and adjust the length of the fiber. Typically, a length of 2 cm is sufficient for most brain regions in mice (Figure 7C).
   e. Close the lid of the fiber cleaver.



Regions	AP	ML	DV
LSv	0.05	-0.65	-3.25
MPN	0.00	-0.33	-4.90
BNSTp	-0.30	-0.80	-3.60
AHN	-1.05	-0.55	-5.15
DMH	-1.75	-0.55	-5.20
VMHvl	-1.70	-0.75	-5.80
PMv	-2.40	-0.55	-5.70
MeAa	-1.10	2.10	-4.90
MeApd	-1.60	2.10	-4.92
PA	-2.35	2.20	-4.92
CoAp	-2.85	2.90	-5.20
SUBv	-3.35	2.60	-4.60

Figure 6. Diagrams show the selected channels in the 48-channel MT ferrules for targeting 12 brain regions in the limbic system

One additional 100-μm optical fiber was implanted at lPAG. Coordinates are on the Bregma (unit: mm). LSv, ventral part of the lateral septum; MPN, medial preoptic nucleus; BNSTp, posterior part of the bed nucleus of stria terminalis; AHN, anterior hypothalamus; DMH, dorsomedial hypothalamus; VMHvl, ventrolateral part of the ventromedial hypothalamus; PMv, ventral part of the premammillary nucleus; MeAa, anterior part of the medial amygdala; MeApd, posterodorsal part of the medial amygdala; PA, posterior amygdala; CoAp, posterior cortical amygdala; SUBv, ventral subiculum; lPAG, lateral periaqueductal gray. Adapted from Ref.[1]

f. Move the blade back and forth multiple times to scratch and break the coating. Then, push the blade all the way to the end to cleanly cleave the fiber.
g. Use a small piece of tape to pick up the cleaved fiber carefully (Figure 7D).
h. Inspect the cleaved end of the fiber under a 5x microscope (e.g., Stemi 2000, ZEISS) to ensure it appears smooth and even, like a mirror. We call this end the smooth end (Figure 7E). Problem 2.

Note: The outer-layer coating of the fiber may experience minor breakage, but this typically does not affect recording quality.

i. Insert the prepared fiber into the target channel of the MT ferrule, making sure the smooth end of the fiber is inserted into the solid end of the MT ferrule (Figures 7E–7G).
j. Repeat steps **2c-i** for the remaining fibers until all fibers are inserted into the corresponding channels.

3. Fiber alignment.
   a. Use the longest protruding fiber, which records the most ventrally situated brain region, as the reference channel.
   b. Calculate the difference in length between the reference channel and all other channels (Figure 8A).

⚠ CRITICAL: Account for the thickness and curvature of the mouse skull by adding an additional 0.5–1 mm in length to all the channels. For example, if the deepest recording site is located at 5.5 mm below the surface of the skull, the longest optic fiber should protrude below the hollow end of the MT ferrule by 6–6.5 mm.

   c. Attach the fiber-loaded MT ferrule to a holder and attach the holder to a stereotaxic frame (Figures 8D and 8E).

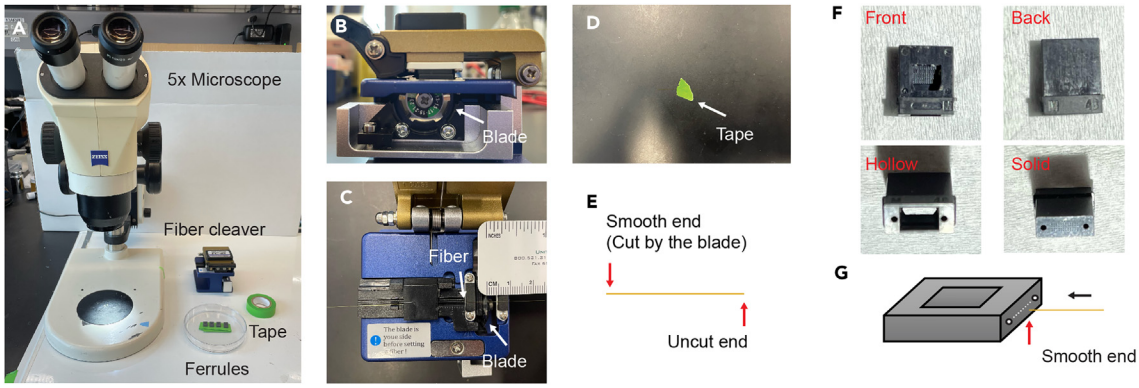


Figure 7. Key procedures for multi-fiber array assembly

(A) Tools for assembling multi-fiber arrays.

(B) Adjust the blade of the fiber cleaver to find a suitable position for 100-μm fiber.

(C) Load the fiber onto the fiber cleaver and measure the length of the fiber.

(D) Use a small piece of tape to handle fiber.

(E) Schematic illustration of the 100-μm fiber after cutting.

(F) Images representing different sides of the MT ferrule.

(G) Insert the fiber into a selected channel. Note the insertion direction.

Note: In our case, we taped an MT ferrule to a Kopf glass pipette holder (Model 1975) to hold the fiber-loaded MT ferrule. The glass pipette holder is an attachment of the Kopf stereotaxic system (Model 1900).

⚠ CRITICAL: Avoid leaving too long excess fibers as they may be stuck on the tape during the adjustment process. Trim the fibers before attaching the MT ferrule for adjustment (Figure 8B).

d. Secure a 200-μm optic fiber (e.g., R-FOC-L200C-50NA, RWD Life Science) on a stand. This single fiber will be used to adjust the protruding length of each 100-μm fiber within the MT ferrule (Figures 8C and 8E).

e. Carefully lower the fiber-loaded MT ferrule until the tip of the 200-μm optical fiber makes contact with the bottom edge of the MT ferrule (Figure 8F). Set the z-axis to zero.

f. Raise the fiber-loaded MT ferrule until the bottom edge of the ferrule is at a distance equivalent to the desired length (e.g., 6.5 mm) of the reference fiber (the longest fiber in the array). Zero the z-axis once again (Figure 8G).

g. Raise the MT ferrule a bit more until all pre-loaded 100-μm fibers are above to the tip of the 200-μm fiber.

h. Move the x- and y-position of the MT ferrule and place the reference fiber above the 200-μm optical fiber.

i. Gently lower the reference fiber until it reaches the zero point on the z-axis, meaning that the fiber is at its desired length (Figure 8G).

⚠ CRITICAL: Ensure that the 200-μm fiber points straight upward and its surface is flat and clean to prevent damage to the smooth end of the 100-μm fiber in the multi-fiber array.

j. Position each 100-μm fiber above the 200-μm fiber tip and lower the 100-μm fiber to the z-axis coordinate equivalent to the length difference between the fiber being lowered and the reference fiber, as calculated from step **3b.**

Note: During the lowering process, the 200-μm optic fiber pushes the 100-μm fiber upward (Figures 8G and 8H). At the end of the process, all fibers should be at their desired length.

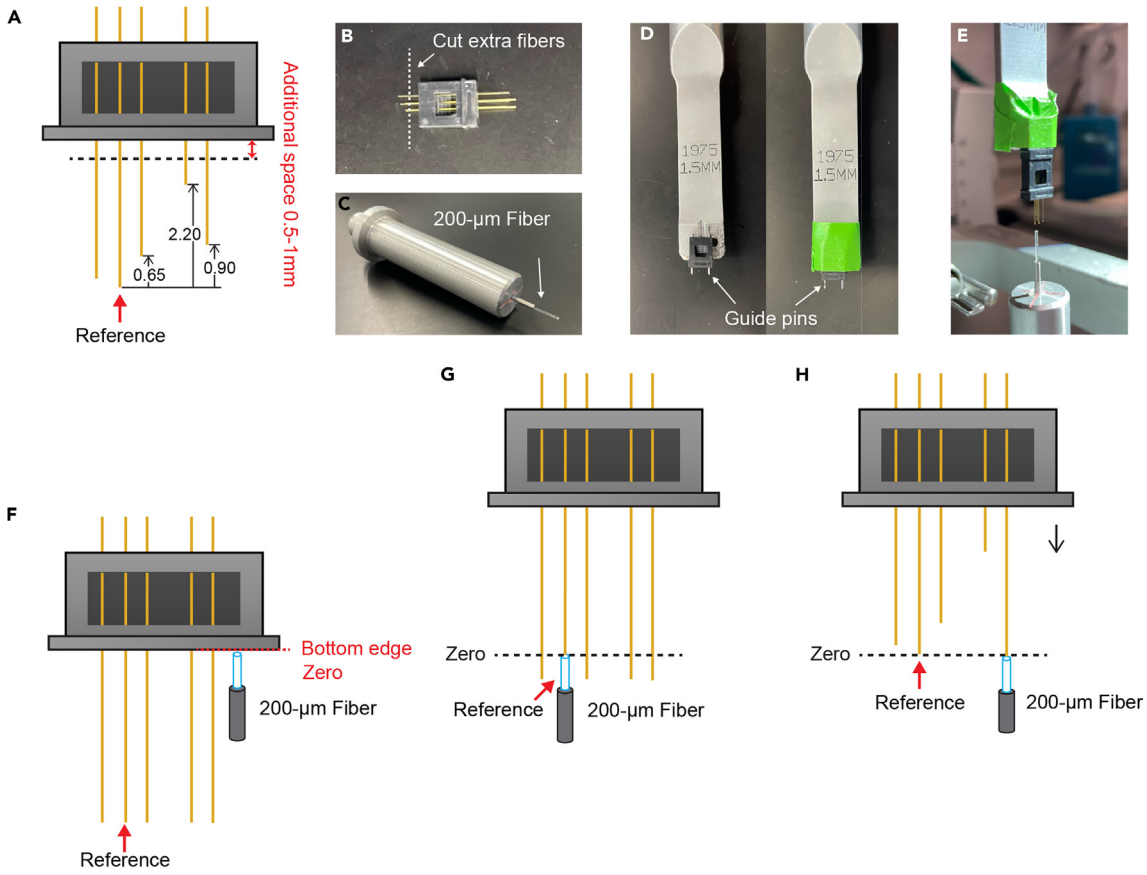


Figure 8. Key procedures for multi-fiber array alignment

(A) Select the reference channel and calculate the difference between the reference channel and other channels.

(B) Trim extra fibers before attaching the unfinished multi-fiber array to the holder.

(C) A 200 μm optical fiber ferrule assembly on a stand.

(D) A ferrule holder made of an MT ferrule with two guide pins.

(E–H) Adjust the depth of the fibers according to the reference fiber by pushing these fibers upward using the 200-μm optical fiber.

k. Carefully detach the ferrule from the holder after completing the fiber length adjustment.

l. Store the fiber-loaded MT ferrule in a petri dish by placing it on a piece of tape or clay (Figure 9A).

4. Glue the fibers to the MT ferrule.

a. Mix the two-part epoxy adhesive (AB9112-2.5G, Fiber Optic Center) thoroughly (Figure 9B).

Note: If the curing adhesive has been stored for an extended period and contains solid fragments, gently warm it at 50°C for approximately 2–5 minutes. This should dissolve all the fragments.

b. Withdraw an appropriate amount of epoxy using a 1 mL syringe (BD30659, Fisher Scientific).

c. Attach a 22-gauge or larger needle (BD305155, Fisher Scientific) to the syringe (Figure 9C).

d. Hold the needle tightly and slowly inject the epoxy adhesive into the front side of the MT ferrule until the hole on the hollow end is filled (Figure 9D).

e. Apply a drop of epoxy to the solid end of the MT ferrule, ensuring that the drop covers all the fibers (Figure 9E). This step helps prevent damage to the fibers during the fiber polishing process.

⚠ CRITICAL: Ensure that the epoxy does not enter the two large holes in the smooth end designed to connect the MT ferrule with the matching ferrule of the recording patch cord (Figure 9F).

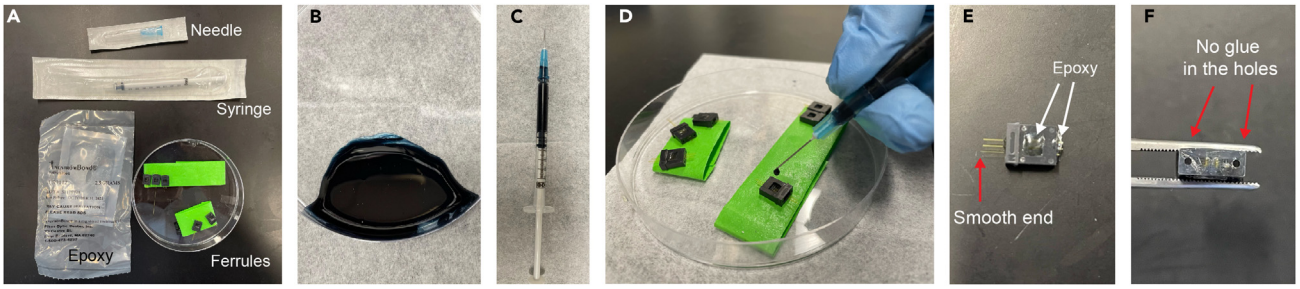


Figure 9. Key procedures to bond the optical fibers with the MT ferrule

(A) Required tools and materials.
(B) Mixed epoxy in a Petri dish.
(C) A syringe filled with the mixed epoxy.
(D) Fill the multi-fiber array with the mixed epoxy from the front side of the ferrule.
(E) A ferrule with epoxy at both front and solid sides.
(F) The holes for guiding pins should be free of epoxy.

Pause point: Wait for 24 hours until the epoxy solidifies completely.

5. Fiber polishing.
   a. Prepare the necessary materials as shown in Figure 10A and Table 1.
   b. Trim excess epoxy and fibers from the ferrule using a razor blade or scissors (Figure 10B).
   c. Load the MT ferrule onto the 3D-printed polishing disk (Figure 10C).

   Note: After multiple uses, the polishing disk may lose its grip on the ferrule. You can print a new polishing disk or secure the ferrule by adding a drop of cyanoacrylate glue (e.g., Krazy glue) to the side.

   d. Hold the ferrule with a small, serrated clamp (Hartman Hemostats, Cat# 13003-10, Fine Science Tools) (Figure 10D).

   Note: As the ferrule is made of plastic, protect it during the polishing process by covering the teeth of the clamp with tape (Figure 10D).

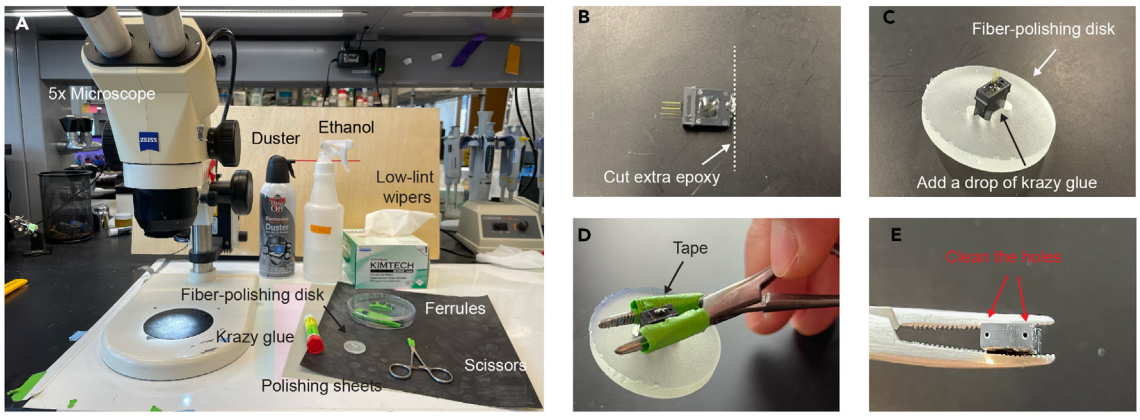


Figure 10. Key procedures for polishing the multi-fiber array

(A) Tools and materials for polishing multi-fiber array.
(B) Trim excess epoxy and fibers at the solid side of the MT ferrule.
(C) A ferrule with a 3D-printed fiber-polishing disk.
(D) Use a small serrated clamp to hold the MT ferrule.
(E) Clean the holes during fiber polishing.

Table 1. Materials required for fiber polishing	
Materials	
Zeiss Stemi 2000 Stereo Microscope	Ethanol
Duster	Fiber-polishing disk
Krazy glue	MT Ferrules with fibers
Low-lint wipers	Polishing Sheets, 5 μm Grit
Serrated clamp	Polishing Sheets, 1 μm Grit
3M Wetordry Sandpaper, P400	Polishing Sheets, 0.3 μm Grit

e. Polish the ferrule using P400 sandpaper (Wetordry Sandpaper, P400, 3M) placed on a flat solid surface (Figure 10D).

⚠ CRITICAL: Regularly clean the debris with ethanol and inspect the solid end of the MT ferrule to ensure that you only remove the excess epoxy, not damaging the ferrule itself.

f. When only a thin layer of epoxy remains, clean the surface with ethanol and low-lint wipers. Then, use a compressed air duster (e.g., Falcon Dust-Off) to remove debris stuck in the holes (Figure 10E).
g. Continue polishing the ferrule using a 5-μm-grit polishing sheet (LF5P, Thorlabs) until all excess epoxy is removed.
h. Clean the surface with ethanol and ensure the holes are clear.
i. Apply ethanol to a 1-μm-grit polishing sheet (LF1P, Thorlabs) and continue polishing the ferrule. Stop when the surface becomes smooth and reflective.
j. Clean the surface with ethanol and ensure the holes are clear.
k. Apply ethanol to a 0.3-μm-grit polishing sheet (LF03P, Thorlabs) and continue polishing the ferrule to further refine the surface.

Assemble and polish the matching patch cord

Timing: 2 days

This section describes the procedures to assemble a multi-fiber patch cord that matches the multi-fiber array.

6. Slide the boot (Boot_48F MT, Cat#: 12599, US Conec) of the MT ferrule onto the custom patch cord (Doric Lenses, see Supplementary materials for ordering information) (Figure 11A).
7. Insert each fiber of the patch cord into a targeted channel of the MT ferrule (Ferrule_48F MM MT, Cat#: 12599, US Conec) from the ferrule's hollow end. This procedure should be done under a microscope. Using tweezers with fine tips can ease the insertion process (Figures 11A and 11B).
8. Trim excess fibers and connect the MT ferrule with the boot (Figure 11C).
9. Follow the same procedures outlined in steps **4** and **5** of the "assemble multi-fiber array" section to secure the fibers and polish the solid end of the patch cord-attached MT ferrule (Figures 11D–11F).

Surgery and multi-fiber array implantation

Timing: 6 h

This section outlines the procedures for virus injection and multi-fiber array implantation.

10. Anesthetize the mouse using an appropriate concentration of isoflurane and place it in a stereotactic frame.

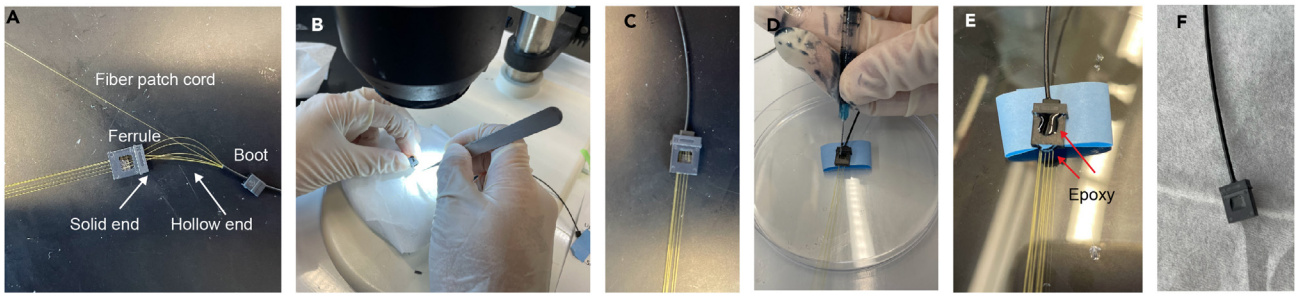


Figure 11. Key procedures for patch cord assembly

(A) A patch cord with loose ends attached to a boot and an MT ferrule.

(B) Insert individual fibers of the patch cord into selected channels of the MT ferrule under a 5× microscope.

(C) Connect the MT ferrule with the boot.

(D and E) Apply a drop of epoxy to the front side and the solid end of the MT ferrule.

(F) Fiber patch cord after trimming excess fibers and polishing.

⚠ CRITICAL: It's crucial to continuously monitor the mouse's breathing throughout the surgery. We recommend gradually reducing the isoflurane concentration from 1.5% to 1% over the course of the surgery to prevent over-anesthetizing the animal.

11. Open the skin to expose the skull surface and ensure it is leveled (Figure 12A).
12. Drill holes in the skull using a small drill bit (e.g., #79, 10WS79-550FL, Drill Bit City) according to the coordinates of the target regions. In our case, we used Kopf Model 1911 Stereotaxic Drill (Figures 12B and 12C).
13. Slowly (e.g., flow rate: 20 nL/min) inject a small amount of virus (e.g., 80 nL AAV1-CAG-Flex-GCaMP6f-WPRE.SV40, 100835-AAV1, Addgene) into each brain region of interest using a microinjector (e.g., Nanoliter 2020 injector, WPI). Adjust the volume and flow rate as needed for specific brain regions.
14. After the injection, keep the injection needle in place for at least 5 min to allow the virus to diffuse, and then slowly retract the needle.
15. Repeat steps **13–14** to inject the virus into all selected brain regions.
16. Attach the assembled multi-fiber array to the holder (Figure 12D).
17. Use the longest fiber to position the multi-fiber array. Once the longest fiber is properly positioned into the corresponding hole on the skull, all other fibers should fit snuggly into the pre-drilled holes (Figure 12E). Problem 3.
18. Implant the multi-fiber array approximately 200 μm above the depth of virus injection.
19. Secure the implanted multi-fiber array on the skull surface using dental cement (e.g., C&B Metabond quick adhesive cement system, Cat# S380, Parkell) (Figure 12F).
20. Place a head-fixation ring on the skull and secure it with dental cement (Figure 12G).

⏸ **Pause point:** Wait three weeks for the virus expression.

MFP recording

⏱ **Timing: 2 h**

This section outlines the steps to set up the multi-fiber recording system and record the signal.

21. Open StreamPix 8 (other versions will work equally well) or your preferred recording software. Load the camera for MFP recording. Set the camera's image format to Mono8 to capture images in grayscale.
22. Connect the recording patch cord to the MFP setup (Figure 13A).

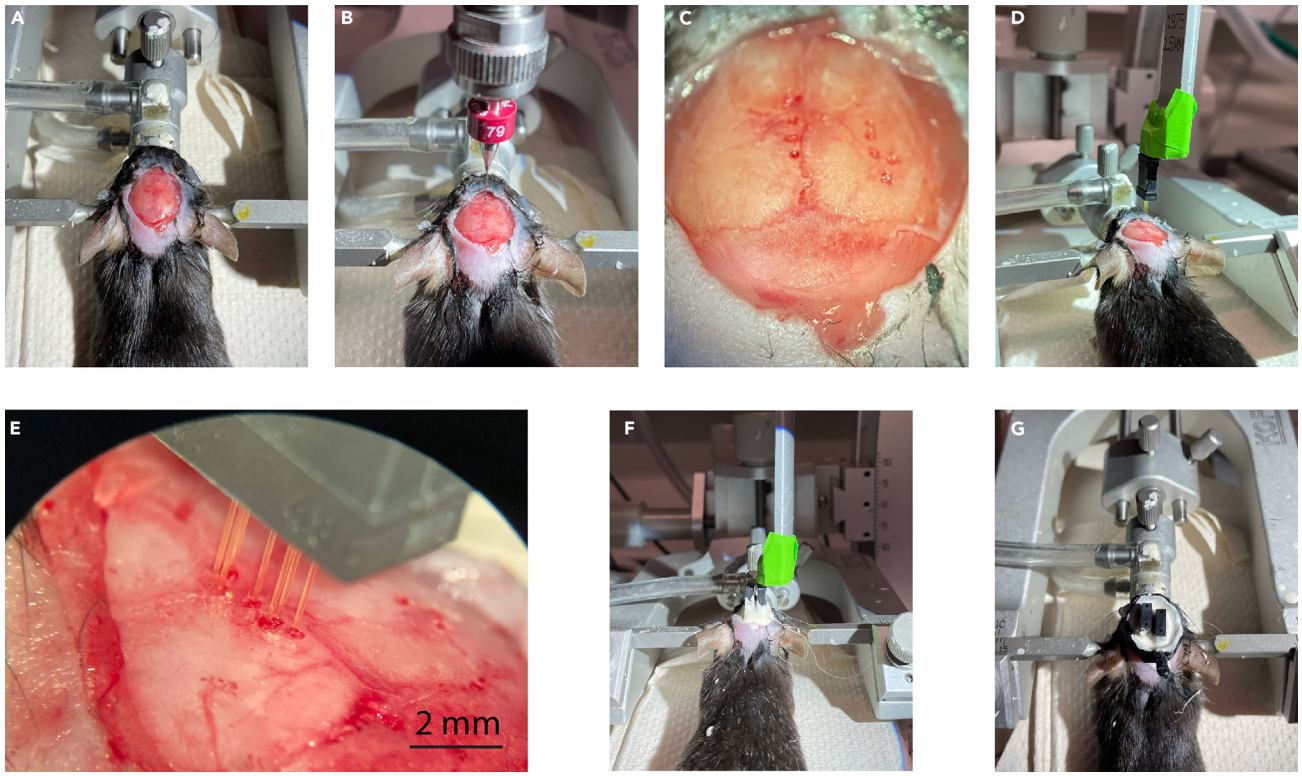


Figure 12. Multi-fiber array implantation during surgery

(A) Open the skin to expose the skull surface.

(B and C) Drill holes in the skull using a small drill bit.

(D and E) Attach the assembled multi-fiber array to the holder and insert the fibers into the drilled holes on the skull. Scale bar, 2mm.

(F and G) Secure the implanted multi-fiber array using dental cement and place a head-fixation ring on the skull.

23. Point the unconnected end of the patch cord (with the MT ferrule) towards the room light. Adjust the SMA fiber adapter plate along the rods of the 30 mm cage system (Figure 13A) until the fiber ends of the patch cord are in focus (Figures 13B and 13C).

Note: If the fiber bundles' image is not centered in the frame, adjust the screws on the XY translator that is directly connected to the camera for proper alignment.

24. Turn on the LED power supply and switch it to "CW" mode to output constant excitation light.
25. To measure the output power of the recording ferrule, use a power meter (models SM1T2 and S120C from Thorlabs).
    a. Set the power meter's range to 800 μW and the wavelength to 470 nm.
    b. Cover the sensor with the lip to zero the meter.
    c. After this calibration, direct the recording ferrule towards the sensor to obtain the power reading.
26. Adjust the power supply of the LED to ensure that the light intensity of each channel is around 30 μW. The overall output power should equal the number of channels multiplying 30 μW.

Note: If the total light output is below the desired power level even when the power supply is at its maximum output, consider adjusting the screws on the XY translator connected to the LED. This adjustment can help better align the excitation light and patch cord for optimal performance.

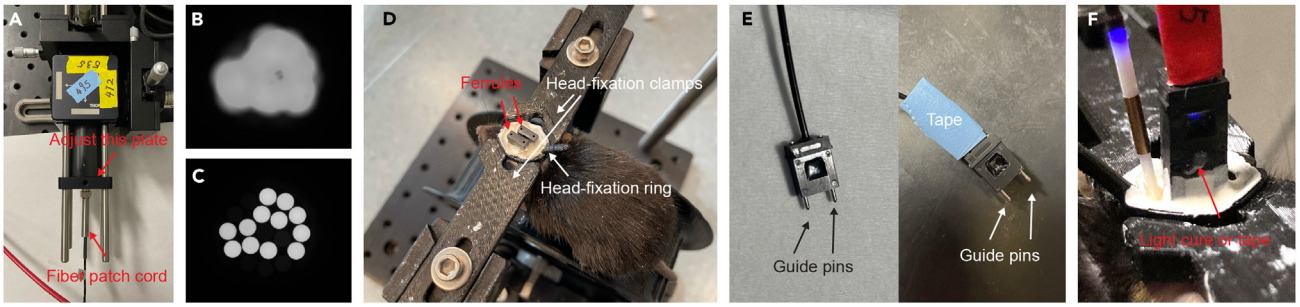


Figure 13. Key procedures for MFP recording

(A) Adjust the position of the SMA fiber plate until the recording patch cord end is in focus.

(B) A blurry view of the recording patch cord end before adjustment.

(C) A clear view of the recording patch cord end after adjustment.

(D) Head-fixed mouse on a running wheel.

(E) The patch cord-attached MT ferrule with two inserted guide pins and a piece of blocking tape.

(F) Connect the recording patch cord with the implanted multi-fiber array.

27. Again, point the recording ferrule towards room light. Block each channel using a fine needle to match the position of the fiber end in the image to the fiber position in the MT ferrule, which determines the targeted brain region.

⚠ CRITICAL: Steps 23–27 need to be carried out whenever the patch cord is reconnected to the MFP system.

28. Secure the head of the mouse using the 3D-printed head-fixation system (Figure 13D).
29. Insert two flat-end guide pins (Cat#12766, US Conec) in the MT ferrule attached to the patch cord and use a piece of tape to block the movement of the guide pins (Figure 13E).
30. Clean the surface of the implanted multi-fiber array and the patch cord-attached MT ferrule with ethanol and low-lint wipes.
31. Connect the patch cord with the implanted multi-fiber array (Figure 13F).
32. Adjust the exposure time and the gain of the camera to ensure the light intensity of all channels falls within the dynamic range of the camera.

Note: For our recordings, we typically set the exposure time to 20–30 ms, with a gain ranging from 250 to 400. It is important to note that a high-gain setting may result in a noisy background. In the Mono8 format, each pixel's value ranges from 0 to 255. Ideally, the light intensity for all channels at the baseline falls between 30 and 100. However, achieving a similar intensity level across all channels is often challenging due to variability in virus expression and fiber placement. Therefore, empirically, any baseline signal ranging from 15 to 180 is acceptable.

33. The frame rate of the video should be set based on the temporal dynamics of the fluorescence signal. For GCaMP recording, 25 frames/s is sufficient.
34. Secure the connection using an accelerated light cure (e.g., Flow It ALC Flowable Composite, Henry Schein) or tape (Figure 13F) to ensure the connection is stable.
35. Place the mouse in the recording chamber, begin the behavior tests, and record.

⚠ CRITICAL: The MFP signal can be affected by bright room light (infrared or visible). Low room light intensity is recommended.

Data extraction

Timing: 1 h

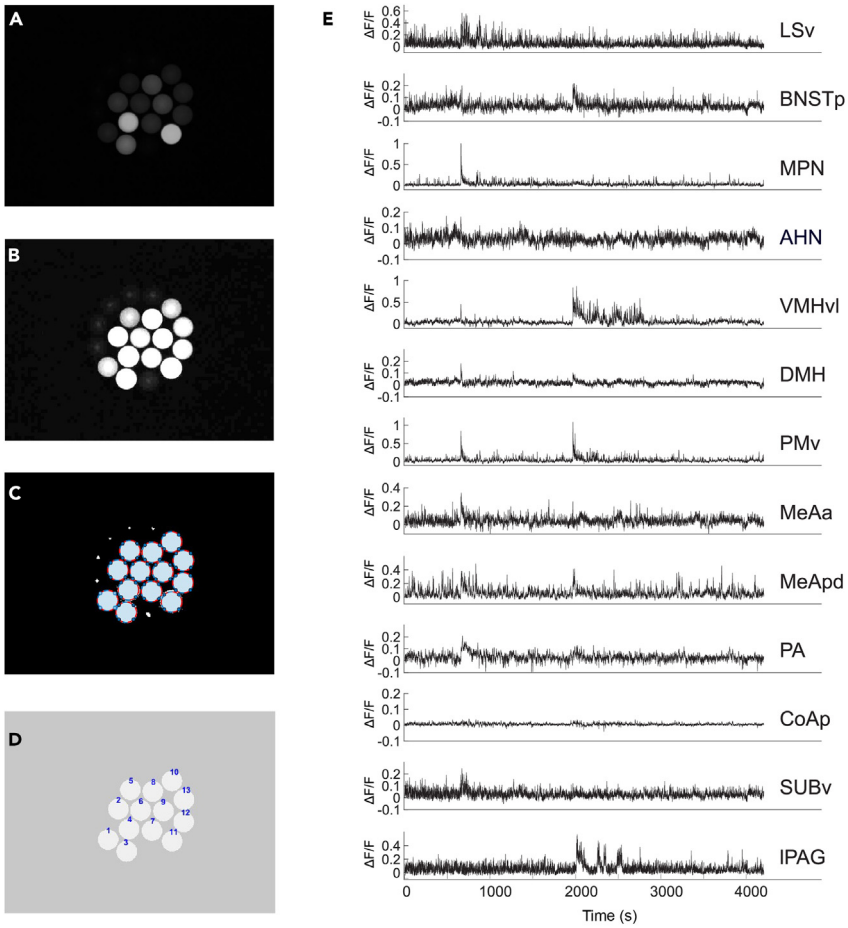


Figure 14. Data extraction from an example dataset and data illustration

(A) An example frame from a video showing the ends of a multi-fiber recording patch cord.

(B) The example frame after adjusting the contrast.

(C and D) Multiple binary masks (C) that are generated based on the example frame in (A and B) and (D) The order of the masks.

(E) Simultaneously recorded GCaMP6f traces (ΔF/F) from the example dataset. IPAG was recorded using a separate single optic fiber. Adapted from Ref.[1]

This section describes the procedures to extract the fluorescence signals from the recorded videos in MATLAB.

36. Download the Piotr's Computer Vision MATLAB Toolbox Version 3.50 from https://pdollar.github.io/toolbox/ for processing .seq video file. Problem 4.
37. Download the vislabels function, written by Steve Eddins, from https://www.mathworks.com/matlabcentral/fileexchange/19665-visualize-output-of-bwlabel for visualizing masks.
38. Download the MFR_Examples file from Supplementary materials.
39. Add the code and dependencies into MATLAB path.
40. Open MFR_Mask_for_mp4.m in MATLAB if your video format is mp4. Open MFR_Mask_for_-seq.m if your video format is seq.
41. In the MATLAB code, you need to specify the pathway of the recorded video.
42. Run the code to generate a mask for data extraction automatically. The code extracts the 1st frame of the video (Figure 14A), adjusts the contrast of the image (Figure 14B), and generates a binary mask for extracting signal from each channel (Figures 14C and 14D).

Note: You can give each channel a name (e.g., brain region) for easy identification problem 5. By default, the channels are named "Channel 1", "Channel 2" … in the MATLAB code.

Note: The mask only needs to be generated once for each recording patch cord as long as the patch cord keeps connected with the MFP setup.

43. Open MFR_Extraction_for_mp4.m in MATLAB if your video format is mp4. Open MFR_Extraction_for_seq.m if your video format is seq.
44. The MATLAB code in step **43** will generate a MATLAB Data file containing an averaged recorded signal for each ROI of each video frame. The ROIs are based on the automatically generated masks (Figure 14D). Recorded signals of all channels will be plotted as MATLAB figures for visualization (Figure 14E).
45. Use the extracted signals for various analyses, such as constructing post-event histograms, calculating the mean responses, or examining correlations across regions. See Ref 1 for example analyses and the relevant MATLAB code.

⚠ CRITICAL: When calculating the dF/F (stored in Lfilter), we utilize the msbackadj function with a 10-second window to correct for photobleaching effects. However, it's important to note that this method might also remove slow transient signals on the scale of minutes. If preserving and analyzing these slow transients is critical for your study, we recommend using the raw data (stored in LMag) for further analysis to avoid loss of these slower signal dynamics.

EXPECTED OUTCOMES

The multi-fiber photometry described in this protocol provides a simple and low-cost solution for simultaneously recording neural activities from tens of brain regions in freely moving animals. Even though the patch cord contains tens of fibers, thanks to the small diameter of individual optic fibers (100 μm), the patch cord is sufficiently lightweight and flexible and does not severely hamper the animal's movement. In our experience, the recording animal has no trouble carrying out various social behaviors, including attacking and mounting a conspecific. However, the patch cord could get tangled over time since no communicator is currently available for the MFP system. Thus, the recording time should be limited to 1–2 h, and the experimenter should monitor the recording closely. During recording, fluctuating light intensity at fiber ends can often be observed. The ΔF/F of the recorded signal is comparable to that obtained using single-channel fiber photometry.

LIMITATIONS

Three are several limitations of the current protocol: First, different brain structures may exhibit variations in cell density and viral tropism preferences, necessitating adjustments in viral serotype, virus titer, and injection volume to achieve consistent baselines across different regions. Targeting different cell types in one test animal is challenging, often requiring extensive breeding efforts and complex virus strategies. Second, spatial constraints are imposed by the pre-determined configuration of MT ferrules, potentially restricting access to certain combinations of brain structures. Third, the expression of fluorescent proteins, such as GCaMP, may not be limited to the soma. Axons may also contain a significant amount of fluorescent proteins. Consequently, the recorded signal of each channel could be contributed by cells in the targeted region as well as inputs originating from infected cells in other regions. Fourth, the surgery time for MFP implantation is long mainly due to one-by-one virus injection into many brain sites. This may be circumvented by expressing the sensor using transgenetic animals. For example, specific Cre driver lines crossed with Cre-dependent GCaMP reporter lines (e.g., Ai95D) can be used to express GCaMP in cells of interest throughout the brain. This approach was not used in our study[1] because we found it difficult to obtain healthy $Esr1^{Cre}$:Ai95D adult mice. Fifth, the current Multi-Fiber Photometry (MFP) system is equipped with a single 470-nm channel and lacks a 405-nm reference channel, which may restrict its correction capabilities of movement artifacts. Users can express GFP in a brain region outside the interested areas and record this to reveal signal fluctuation caused by movement or other artifacts. For guidance on

incorporating a 405-nm reference channel, users can refer to the methodology outlined in Sych et al. study,[5] which details the light path for setting up the 405-nm reference channel.

TROUBLESHOOTING

Problem 1

US Conec's available ferrules may not meet the experiment's requirements. Related to Step 1b.

Potential solution

Custom ferrules can be designed based on the ROIs and printed using a 3D printer. The holes in the array can also be created using a drill press. We have successfully designed and produced ferrule for 200-μm optic fibers but found it difficult to produce ferrules for 100-μm fibers due to the limitation of available drill bits. It is worth mentioning that a custom 200-μm multi-fiber array will produce larger tissue damage. The patch cord comprising 200-μm optic fibers is also stiffer and heavier than that containing 100-μm optic fibers and thus can constrain the recording animals' movement more. If 200-μm arrays are used, the number of recording sites should not exceed 12 in one animal.

Problem 2

Bad fiber quality after cutting. Related to Step 2h.

Potential solution

- The top edge of the blade may carry debris after repeated cutting. Gently clean the blade by using ethanol and low-lint wipes.
- The top edge of the blade can also become blunted. Rotate the blade to utilize the entire length of the blade.

Problem 3

100 μm fibers are thin and pliable. The fibers targeting deep brain regions can sometimes curve slightly. Related to Step 17.

Potential solution

Before cutting, hanging the fibers vertically with a weight (~20 g) can help straighten the fiber (Figure 15).

Problem 4

Videos show snow-like noise instead of patch cord end. Related to Step 36.

Potential solution

The original toolbox in Piotr's Computer Vision MATLAB Toolbox is outdated and incompatible with MATLAB 2023a. We have updated the code. Use the "behaviorAnnotatoin" folder in the Supplementary materials of the current protocol.

Problem 5

Generated masks miss some of the targeted channels. Related to Step 42.

Potential solution

If a channel is too dim, the algorithm may fail to detect it automatically. To prevent this, illuminate all the targeted channels by pointing the end of the recording patch cord to a light source before connecting the recording patch cord to the test animal. Record a brief video, and use this video to generate the mask.

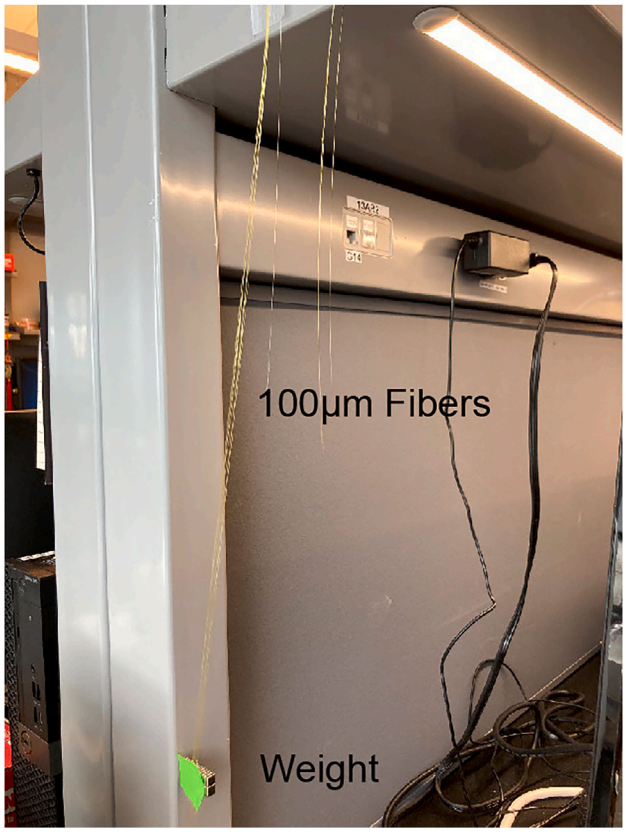


Figure 15. Stretching the 100-μm fibers with a weight

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dayu Lin (dayu.lin@nyulangone.org).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Bing Dai (bd1409@nyu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The Supplementary materials, code, and example dataset generated during this study are available at Zenodo: https://zenodo.org/doi/10.5281/zenodo.8428784.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

D.L. designed the modified MFP system, edited the paper, and supervised the project. B.D. refined the experimental procedures, generated the figures, and wrote the paper. Z.G. constructed the MFP system, refined the experimental procedures, and wrote some instructions.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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