STAR Protocols

Protocol

Protocol for wireless deep brain stimulation in freely behaving mice with infrared light

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Highlights

Protocol for wireless NIR-II deep brain stimulation in freely moving mice

Stereotaxic injection of TRPV1-expressing virus and MINDS into the mouse brain

Steps to perform conditioned place preference test and immunohistochemical analysis

Potential applications for neuromodulation in social interaction experiments

Here, we present a protocol for deep brain stimulation in freely behaving mice using through-scalp wide-field illumination in the second near-infrared window (NIR-II). We first describe the injection of the TRPV1 (transient receptor potential cation channel subfamily V member 1)-expressing viruses and macromolecular infrared nanotransducers for deep brain stimulation (MINDS). We then detail NIR-II neuromodulation in a conditioned place preference test, followed by immunohistochemical studies. This approach is especially useful for tether-free deep brain stimulation in social interacting experiments involving multiple subjects.

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

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Protocol for wireless deep brain stimulation in freely behaving mice with infrared light

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https://doi.org/10.1016/j.xpro.2022.101757

SUMMARY

Here, we present a protocol for deep brain stimulation in freely behaving mice using through-scalp wide-field illumination in the second near-infrared window (NIR-II). We first describe the injection of the TRPV1 (transient receptor potential cation channel subfamily V member 1)-expressing viruses and macromolecular infrared nanotransducers for deep brain stimulation (MINDS). We then detail NIR-II neuromodulation in a conditioned place preference test, followed by immunohistochemical studies. This approach is especially useful for tether-free deep brain stimulation in social interacting experiments involving multiple subjects. For complete details on the use and execution of this protocol, please refer to Wu et al. (2022).

BEFORE YOU BEGIN

Neuromodulation techniques are powerful tools for dissecting complex neural circuitry and potentially treating neurological disorders (Fenno et al., 2011; Jiang et al., 2022; Montgomery et al., 2015; Tsai et al., 2009). Current popular electrical and optical neuromodulation techniques, however, require invasive implantation of the stimulation electrode or optical fiber, which inevitably leads to acute brain damage, chronic gliosis, and physical tethering. Despite recent advances in novel neuromodulation techniques (Chen et al., 2015; Chen, 2018; Kim et al., 2013; Wu et al., 2019), no existing optical methods can eliminate both brain implants and head tethering altogether.

Here we introduce a detailed protocol of using brain-penetrant NIR-II light to activate deep-brain neurons with a tether-free and implant-free interface (Wu et al., 2022). Specifically, this protocol describes the specific steps for NIR-II neuromodulation of midbrain dopaminergic (DA) neurons in the ventral tegmental area (VTA) of mice. Additionally, we have also used this protocol for tether-free NIR-II neuromodulation in the mouse motor cortex and hippocampus.

Institutional permissions

All procedures performed on the mice were approved by Stanford University's Administrative Panel on Laboratory Animal Care (APLAC). The animal care and use programs at Stanford University meet the requirements of all federal and state regulations governing the humane care and use of laboratory animals, including the United States Department of Agriculture (USDA) Animal Welfare Act, and the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. The laboratory animal care program at Stanford is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC International). The readers should acquire permission from the relevant institutions before carrying out any procedures in this protocol.

Adeno-associated virus (AAV) solution preparation

⏱ Timing: 30 min

1. Upon arrival of the AAV solution, thaw the viral stock on ice inside a biosafety cabinet (BSC). Check the viral titer on the product sheet.

   Optional: Dilute the virus using ice-cold 1× PBS as needed. The viral titer used in this protocol is 3.6 × 10^{12} GC/mL. If the viral stock has the desired titer, no dilution is needed.

2. Aliquot 10 μL of the AAV solution into a low protein binding tube.

   Note: The low protein binding tube helps prevent the gradual loss of the virus when the AAV solution is stored for extended periods of time.

3. After the entire solution is aliquoted, store the aliquots at −80°C until use.

MINDS preparation

⏱ Timing: 2 days

4. Weigh and dissolve reactants.
   a. Weigh 25 mg (0.023 mmol) of 4,7-bis(5-bromo-4-(2-octyldodecyl)thiophen-2-yl)bisbenzothiadiazole, 0.6 mg (0.00066 mmol) of tris(dibenzylideneacetone)dipalladium(0) and 1.8 mg (0.006 mmol) of tri(o-tolyl)phosphine.
   b. Transfer the chemicals in step 4a into a 50 mL Schlenk flask.
   c. Add 16 μL (0.03 mmol) of trans-1,2-bis(tributylstannyl)ethene, and add 8 mL of chlorobenzene for dissolution.
5. Complete three freeze-pump-thaw cycles.
   a. Seal the mixture in a Schlenk flask under N_2.
   b. Put a Dewar with liquid nitrogen underneath the Schlenk flask to freeze the mixture inside.
   c. Open the stopcock of the Schlenk flask and keep it under vacuum for 5 min. Keep the flask immersed in liquid nitrogen.
   d. Close the stopcock and remove the liquid nitrogen.
   e. Repeat the above procedures for two more times.
6. Heat up the mixture inside the Schlenk flask to 100°C in an oil bath in N_2 atmosphere to perform Stille polycondensation. React for 100 min.
7. Afterwards, add the mixture dropwise to ice-cold methanol under stirring.
8. Centrifuge the mixture at 9,000 rpm (9,418 × *g*) for 10 min at 0°C and collect dark precipitates.
9. Wash the precipitates three times by resuspending them in ice-cold methanol followed by centrifugation at 9,000 rpm (9,418 × *g*) for 10 min at 0°C.
10. Dry the precipitates under vacuum to obtain purified poly(benzobisthiadiazole-*alt*-vinylene) (pBBTV) powder.
11. Weigh 0.1 mg of pBBTV and 4 mg of poly(lactide-co-glycolide)-b-poly(ethylene glycol) (PLGA-PEG, PLGA M_n 4500, PEG M_n 2000). Dissolve them by adding 2 mL of tetrahydrofuran (THF).
12. Quickly inject the mixture from step 11 into 10-mL of deionized (DI) water under sonication. A homogenous suspension should be obtained.
13. Gently evaporate THF under N_2 flow for 40 min.
14. Sonicate the remaining solution for 10 min. Then pass the solution through polyvinylidene fluoride syringe filter (220 nm) to remove precipitates. The MINDS solution is obtained.
15. Prepare concentrated stock solution of MINDS.
   a. Centrifuge the MINDS solution in step 14 at 3,500 rpm (1,424 × *g*) at 4°C for 25 min in a centrifugal filter (50 kDa cut-off).

   b. Take the remaining solution inside the centrifugal filters.

16. The concentration of MINDS stock solution can be determined through its optical absorbance measurement.

   a. Take 30 μL of MINDS stock solution and dilute it to 3-mL by adding 2,970 μL of DI water.

   b. Load the 3-mL diluted MINDS solution into a 1-cm cuvette, and measure its absorbance at 1,064 nm using a UV-Vis-NIR spectrometer.

   c. The concentration of the MINDS stock solution can then be calculated as [MINDS] = Abs/0.02358 (μg/mL).

   d. Dilute the stock solution with appropriate amount of 1× HHBS to obtain 1.8 mg/mL MINDS solution for injection.

Note: MINDS solutions can also be obtained from the corresponding author upon reasonable request.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken anti-TH	Abcam	ab76442
Mouse anti-TRPV1	Abcam	ab203103
Rat anti-GFAP	Thermo Fisher Scientific	13-0300
Rabbit anti-Iba1	Wako Chemicals	013-27691
Rabbit anti-Cleaved Caspase-3	Cell Signaling Technology	9664
Goat anti-chicken, Alexa Fluor 647	Abcam	ab150171
Goat anti-mouse, Alexa Fluor 568	Abcam	ab175473
Goat anti-rat, Alexa Fluor 647	Abcam	ab150159
Donkey anti-rabbit, Alexa Fluor 594	Invitrogen	A-21207
AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	115-007-003
Chemicals, peptides, and recombinant proteins		
Ethanol solution, 70%	Fisher Scientific	BP82011
1× PBS	Thermo Fisher Scientific	J61196-AP
1× HHBS	AAT Bioquest	20011
Paraformaldehyde, 16% w/v aqueous solution	Fisher Scientific	AA433689L
O.C.T. compound	VWR	25608-930
Methanol	Sigma-Aldrich	34860-1L-R
Normal goat serum	Jackson ImmunoResearch	005-000-121
Normal donkey serum	Jackson ImmunoResearch	017-000-121
Triton™ X-100	Sigma-Aldrich	X100-100ML
4,7-bis(5-bromo-4-(2-octyldodecyl)thiophen-2-yl)bisbenzothiadiazole	Luminescence Technology Corp.	CS10361
Trans-1,2-Bis(tributylstannyl)ethene	Sigma-Aldrich	731625
Tris(dibenzylideneacetone)dipalladium(0)	Sigma-Aldrich	328774
Tri(*o*-tolyl)phosphine	Sigma-Aldrich	8418170001
Chlorobenzene	Sigma-Aldrich	8017911000
Poly(lactide-*co*-glycolide)-*b*-poly(ethylene glycol)	Sigma-Aldrich	764825
Tetrahydrofuran	Sigma-Aldrich	401757
Ketamine hydrochloride injection (100 mg/mL)	Dechra	N/A
Dexmedesed® (dexmedetomidine hydrochloride) (0.5 mg/mL)	Dechra	N/A
Bacterial and virus strains		
AAV5-EF1a-DIO-TRPV1	Vector Biolabs	AAV-275106

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: C57BL/6J, 6 weeks, male	The Jackson Laboratory	000664
Mouse: B6.Cg-7630403G23Rik$^{Tg(Th-cre)1Tmd}$/ J, 6 weeks, male	The Jackson Laboratory	008601
Other		
Low binding plastic microcentrifuge tubes	Fisher Scientific	07-200-183
Stereotaxic frame	World Precision Instruments	505323
Nanojet stereotaxic syringe pump	Chemyx	10050 & 10051
Neuros syringes (10 μL)	Hamilton Company	65460-04
Dumont #5 fine forceps	Fine Science Tools	11254-20
Vannas spring scissors	Fine Science Tools	15000-08
Disposable scalpel	Fisher Scientific	NC0595256
Stereoscopic microscope	AmScope	SM-3T-54S-5M
Nair hair removal lotion	Amazon	ASIN#B001RVMR7K
Drill machine	Amazon	ASIN#B089NLN4CP
Vet ointment	Amazon	ASIN#B002Z9CFPW
Antibiotic ointment	Amazon	ASIN#B000NQ10FK
Vetbond tissue adhesive	Amazon	ASIN#B004C12Q46
Cotton-tipped applicators	Fisher Scientific	22-025-202
Betadine	Fisher Scientific	19-027132
Gauze sponges	Fisher Scientific	22-415-469
Alcohol pad	VWR	75856-902
Atipamezole	Zoetis	N/A
1064-nm laser	RPMC Lasers	SOL 20W-CW-1064nm-FC
Fiber collimator	Thorlabs	F810SMA-1064
Locking ball and socket mount	Thorlabs	TRB2
Power meter	Thorlabs	S405C
Y maze	Maze Engineers	3501
Virkon S	Amazon	ASIN#B0088OKF1E
Thermal camera	FLIR	A325sc
Video camera	Canon	VIXIA HF W11
Arbitrary function generator	Tektronix	AFG 31000
BNC cable	Amazon	ASIN#B07T28BWN6
IR viewer	Sofradir	ElectroViewer 7215
Syringe pump	Harvard Apparatus	70-4500
Brain Slicer	Braintree Scientific	BS-SS
Cryomold	Electron Microscopy Sciences	62534-15
Extra-long forceps	Fisher Scientific	10-316C
Cryostat	Leica Biosystems	Leica CM 3050S
Paint brush	Princeton Brush Company	Series 9100 (5/0)
Kimwipes	Amazon	ASIN#B075L9ZTPB
Microplate shaker	Fisher Scientific	88-861-023
Superfrost™ plus glass slides	Fisher Scientific	22-037-246
ProLong™ Gold Antifade Mountant	Fisher Scientific	P10144
Nail polish	Fisher Scientific	50949071
Balance	METTLER TOLEDO	XSR105
Schlenk flask	Fisher Scientific	31-501-357
Centrifuge	Thermo Fisher Scientific	Sorvall Legend x1R
Polyvinylidene fluoride syringe filter (220 nm)	Millipore	SLGV013SL
Amicon ultra-15 centrifugal filters (50 kDa)	Merck KGaA	UFC905024
UV-Vis-NIR spectrometer	Agilent	Cary 6000i
Laser scanning confocal microscope	ZEISS	LSM 780

Note: The "4,7-bis(5-bromo-4-(2-octyldodecyl)thiophen-2-yl)bisbenzothiadiazole" is a customized product from Luminescence Technology Corp.. Dexmedesed is just dexdomitor.

MATERIALS AND EQUIPMENT

Ketamine/Dexdomitor cocktail for surgical anesthesia		
Reagent	Final concentration	Amount
Ketamine (100 mg/mL)	16 mg/mL	0.8 mL
Dexdomitor (0.5 mg/mL)	0.2 mg/mL	2 mL
1 × PBS	N/A	Adjust to 5 mL
Total	N/A	5 mL

Note: The cocktail can be stored at room temperature (20°C–25°C) for 1 month.

Ketamine/Dexdomitor cocktail for transcardial perfusion anesthesia		
Reagent	Final concentration	Amount
Ketamine (100 mg/mL)	24 mg/mL	1.2 mL
Dexdomitor (0.5 mg/mL)	0.2 mg/mL	2 mL
1 × PBS	N/A	Adjust to 5 mL
Total	N/A	5 mL

Note: The cocktail can be stored at room temperature (20°C–25°C) for 1 month.

STEP-BY-STEP METHOD DETAILS
Stereotaxic injection of AAV

⊙ Timing: 1 h

This section describes the procedures for stereotaxic injection of AAV into the VTA of the mouse brain. The surgery procedure was adapted from a previous report with revisions (Cetin et al., 2006).

1. Take out the aliquoted AAV from the −80°C freezer. Thaw the virus on ice.
2. Sterilize all surgical tools with an autoclave. Put the sterilized tools on a sterile gauze pad.
3. Anesthetize a mouse.
   a. Weigh and anesthetize a mouse with intraperitoneal injection of ketamine (80 mg/kg) and dexdomitor (1 mg/kg) cocktail.
   b. Put the mouse on a heating pad of a homeothermic monitoring system with the temperature set to 37°C to prevent hypothermia.
4. Verify the degree of anesthesia through toe pinch before starting the surgery.
5. Apply vet ointment on both eyes of the mouse to keep them moist throughout the surgery.
6. Subcutaneously inject buprenorphine SR (1 mg/kg) for analgesia.
7. Subcutaneously inject 0.2 mL of prewarmed saline to prevent dehydration.
8. Remove the hair using the hair removal lotion.
   a. Use an alcohol pad to gently moisturize the hair, then apply the hair removal lotion using a cotton-tipped applicator.
   b. 1–2 min later, remove the lotion with a clean cotton-tipped applicator (troubleshooting 1).
   c. Sterilize the exposed skin with betadine and 70% ethanol, alternating three times.

Note: We recommend thorough depilation of the head and the neck to prevent accidental NIR-II illumination on the fur in later procedures.
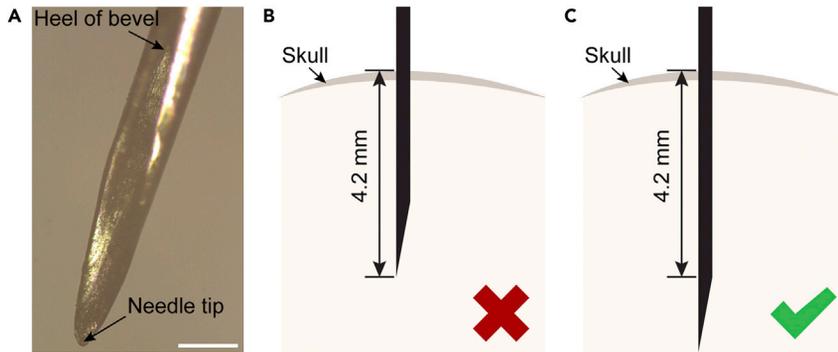
Figure 1. DV positions of the needle
(A) Microscopic image of the needle tip of a Hamilton syringe. The scale bar represents 0.2 mm.
(B and C) The incorrect (B) and correct (C) DV positions of the inserted needle tip.

9. Fix the animal in the stereotaxic frame.
   a. Fix the left ear bar, and gently move the mouse head forward so that the ear canal is close to the ear bar.
   b. Hold the mouse head still, then gradually put the right ear bar in place. The fixing is appropriate if no lateral movement of the head is possible.
   c. Fix the mouth into the anterior adaptor by first pulling down the lower jaw with forceps and then putting the maxillary incisors into the opening of the adapter.
   d. Check the status by gently pressing the head with a cotton-tipped applicator. If no head movement is observed, the fixing is appropriate.
10. Use a sterile scalpel to make a 1-mm incision on the scalp, then use sterilized spring scissors to gradually elongate the incision to expose the skull. Make sure that both bregma and lambda are exposed (troubleshooting 2).
11. Adjust the angle of the head to make it horizontal.
    a. Fix a blunt needle onto the stereotaxic frame and use it to measure the DV positions of bregma and lambda on the skull.
    b. Adjust the angle of the head to make sure bregma and lambda have the same DV positions.
12. Mark the position of VTA on the skull using a sterilized pencil.

    Note: The stereotaxic coordinate of VTA with respect to bregma is AP: -3.5 mm, ML: 0.4 mm.

13. Gently drill a hole (0.5-mm in diameter) on the marked position on the skull. Use a stereoscopic microscope for better visualization and control of the drilling process (troubleshooting 3).
14. Load ∼ 3 µL of AAV solution into the Hamilton syringe and fix the syringe onto the stereotaxic frame.

    Note: For TRPV1 (-) control experiments, a blank AAV solution of the same serotype and titer should be loaded into the syringe instead.

15. Place the tip of the syringe on the bregma and zero the positioning system (troubleshooting 4).
16. Raise the syringe by 5 mm, then move it to AP: -3.5 mm, ML: 0.4 mm. The syringe should now be over the burr hole in the skull.
17. Slowly lower the heel of bevel of the needle to the DV location of VTA (-4.2 mm).

    ⚠ CRITICAL: Since the needle is beveled, there is a distance between the tip and the heel of bevel of the needle (Figure 1A). Make sure the heel of bevel of the needle is lowered to the position of VTA for more precise viral transduction (Figures 1B and 1C).

18. Wait for 5 min after the needle has advanced to the target, and then inject 2.5 μL of AAV solution at a rate of 0.1 μL/min.

    Note: The injection rate can be controlled by the Nanojet stereotaxic syringe pump.

19. After the injection is finished, wait for 2 min before withdrawing the needle to prevent the back-flow of the virus.
20. Release the mouse from the stereotaxic frame.
    a. Put the incised scalp back in place to cover the exposed skull.
    b. Apply Vetbond tissue adhesive to seal the skin.
    c. Apply antibiotic ointment around the wound with a cotton-tipped applicator to prevent infection.

    Note: The Vetbond tissue adhesive acts as an effective means for sealing the scalp, thus requiring no suturing.

21. Subcutaneously inject atipamezole (1 mg/kg) to help accelerate the recovery process.
22. Put the mouse back to the cage, with half of the cage placed on top of the heating pad set to 37°C.

Stereotaxic injection of MINDS

⏱ Timing: 1 h (3–4 weeks after completion of stereotaxic injection of AAV)

This section describes the procedures for stereotaxic injection of MINDS into the VTA of the mouse brain. This step is similar to the previous step "Stereotaxic injection of AAV", and identical procedures will only be briefly described.

23. Prepare the surgical tools as described in step 2. Anesthetize the mouse and fix it in the stereotaxic frame as described in steps 3–11.
24. Find the burr hole in the skull drilled for the previous stereotaxic injection.

    Note: Make sure the burr hole is accessible and large enough for needle insertion. If not, enlarge the hole using a drill according to step 13. Use the stereoscopic microscope for better visualization and control if additional drilling is needed.

25. Load ~ 3 μL of MINDS solution (1.8 mg/mL) into the syringe and inject 2.5 μL into the VTA as described in steps 15–22.

    Note: For MINDS (-) control experiment, 1× HHBS should be injected instead.

Conditioned place preference test

⏱ Timing: 5 days (2 days after completion of stereotaxic injection of MINDS)

This section describes the procedures for conditioned place preference test in mice with NIR-II photothermal neuromodulation in the VTA. The timeline of these procedures is adapted from a previous report (Kim et al., 2013). Experiments have shown that MINDS largely stayed near the injection site, remaining functionally stable over 2 weeks after stereotaxic injection (Wu et al., 2022). Therefore, a waiting period of 2 days prior to the conditioned place preference test is acceptable.

26. Prepare the Y-maze for conditioned place preference test.
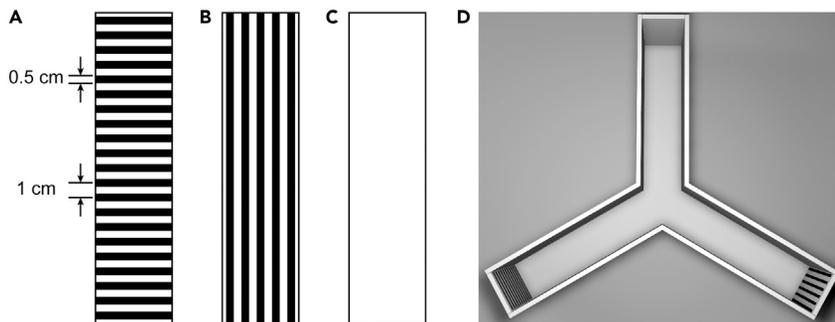
Figure 2. Setup of a Y maze for the conditioned place preference test
(A–C) Schematics showing the vertical strip (A), horizontal strip (B), and blank pattern (C) taped on the three arm terminal walls of the Y maze.
(D) Schematic showing the Y maze with patterns in (A–C) in the three arm terminals.

   a. Cover the bottom of the Y-maze with mouse cage bedding. Make sure that the bedding thickness is similar to that in a regular housing cage.
   b. Print black grating patterns on white paper with 5-mm grating width and 1-cm center-to-center interval between neighboring gratings (Figure 2A). Print the grating patterns in both horizontal and vertical directions (Figure 2B).
   c. Tape the horizontal and vertical grating patterns on the wall of two arm terminals.
   d. For the third arm terminal, tape blank white paper on the wall (Figure 2C).

Note: It is important to also tape blank white paper on the third arm terminal wall, so that all the terminal walls are covered with the same material. Under rare circumstances where the animals damage or scratch the paper, replace the paper immediately after the current session finishes. The paper can be cleaned by gently wiping it with dry Kimwipes for three times between animals.

27. Set up a video camera over the Y maze with sufficient field of view covering the entire Y maze.
28. Set air conditioning to a stable ambient temperature at 25°C.
29. On Day1, run a 30-min pretest.
   a. Place the mouse in the central triangle of the Y maze.
   b. Turn on the video camera to record the trajectory and behavior of the mouse.
   c. Remove the three doors that separate the central triangle and the three arms simultaneously. Let the mouse freely explore the entire Y maze for 30 min.
   d. After the pretest is finished, remove the mouse from the Y maze and put it back to its home cage.

⚠ CRITICAL: Make sure that the environment of the room (including temperature, lighting, humidity, sound, and odor) is stable throughout the pretest. A significant cue of the environment (e.g., loud sound) affects the animal's behavior. We recommend an ambient lighting intensity of $\sim$ 750 lux and a background noise level of $\sim$ 45 dB.

Note: The Y maze can be cleaned with Virkon-S followed by 70% ethanol between animals.

30. On Day2 to Day4, run a 30-min contextual training session with NIR-II neuromodulation each day. The training session and pretest should be performed at roughly the same time of the day.
   a. Connect a fiber collimator to the 1,064-nm laser output fiber.
   b. Adjust the distance between the collimator and the targeted arm terminal of the Y maze to be $\sim$ 1 m, so that the laser spot is circular with a diameter of 1 cm. This 1-m distance also facilitates tracking of the animal with the laser beam.

   c. Fix the fiber collimator on a locking ball and socket mount. Make sure that the angle of the collimator can be freely adjusted to cover a 5 cm × 5 cm area at the end of the targeted arm terminal.
   d. Set up the function generator to output a high voltage (5 V) pulse train with 20 Hz repetition rate and 30% duty cycle.
   e. Connect the output of the function generator to the trigger input of the laser control box via a BNC cable.
   f. Turn on the laser and monitor its output power using a power meter. Adjust the temporal average output power density of the laser spot to be 10 mW/mm^2.
   g. Set up a thermal camera to monitor the temperature of the mouse in the targeted arm terminal of the Y maze.
   h. Place the mouse inside the Y maze, turn on the video camera, and remove the three doors, as described in step 29.
   i. When the mouse enters the 5 cm × 5 cm area at the end of the targeted arm terminal, aim the laser beam to manually track the head of the mouse. Under rare circumstances where the head titling of the mouse prevents aiming the laser beam on the scalp, the laser should be temporally turned off until the head returns to its horizontal position. The 1,064-nm light is invisible to human eyes but can be visualized through an IR viewer.
   j. In the meantime, monitor the temperature on the mouse head through the thermal camera. The laser output is turned down to 0 whenever the scalp temperature is above 39°C and turned back to 10 mW/mm^2 whenever the scalp temperature is below 38°C. The temperature in VTA is estimated to be 39.5°C–39.8°C when the scalp temperature is 39°C (Wu et al., 2022).
   k. Every time when the mouse leaves the 5 cm × 5 cm area in the targeted arm terminal, turn off the laser.

   △ CRITICAL: Make sure to always wear appropriate laser safety goggles when working with lasers.

   △ CRITICAL: Avoid aiming the laser beam on the fur as this may cause damage to the mouse.

   Note: The laser should target the same arm terminal throughout the three training sessions.

31. On Day5, run a 30-min posttest. The procedures for the posttest are identical to those for the pretest, as described in step 29.

   △ CRITICAL: As multiple components (e.g., NIR-II light, MINDS and TRPV1) are involved in this NIR-II neuromodulation technique, appropriate and comprehensive control experiments are needed to rule out artifacts and other confounding factors. We recommend that a control group of mice without TRPV1 transduction (i.e., TRPV1(-), MINDS (+)) and another control group of mice without MINDS injection (i.e., TRPV1(+), MINDS(-)) should go through the same conditioned place preference test, as detailed above. The comparison of the place preference behavior between the pretest and the posttest serves as an internal control for NIR-II light.

   Note: The posttest should be performed at roughly the same time of the day as the training sessions and the pretest.

Immunohistochemical studies of the mouse brain

   ⊙ Timing: 4 days (1 week after completion of conditioned place preference test)

This section describes the procedures for immunohistochemical studies to assess the TRPV1 transduction efficiency and thermal damage associated with the NIR-II neuromodulation. Mice with failed viral transduction (i.e., no TRPV1 expression in VTA) should be excluded from the analysis of data in the conditioned place preference test.

32. 1 week after the posttest of the conditioned place preference test, perform transcardial perfusion to the mouse.
    a. Deeply anesthetize the mouse with intraperitoneal injection of ketamine (120 mg/kg) and dexdomitor (1 mg/kg) cocktail.
    b. Euthanize the mouse through sequential transcardial perfusion of 12-mL 1× PBS and 24-mL 4% PFA (rate: 5 mL/min).

    Note: The perfusion rate is controlled by a syringe pump.

33. Dissect the brain from the skull and immerse it in 15-mL of 4% PFA at 4°C for 24 h.
34. Remove the brain from 4% PFA.
    a. Cut a 3–5 mm thick coronal tissue block including VTA using a brain slicer.
    b. Immerse the brain tissue block into 10-mL of 30% sucrose solution and keep it at 4°C overnight (12–20 h) (troubleshooting 5).
35. Fill a cryomold with O.C.T. compound and transfer the brain tissue block into it. Wait for 30 min.
36. In the meantime, prepare dry ice/methanol slurry in a 10 cm petri-dish.
    a. Make sure to put the petri-dish on top of a foam box cap to keep the temperature low.
    b. Wait until the system equilibrates and add more dry ice if needed.

    Note: At equilibrium, adding new dry ice will cause no or little bubbling.

    ⚠ CRITICAL: Make sure to wear thermally-insulated gloves when working with dry ice.

37. Transfer the brain into a new cryomold filled with O.C.T. compound.

    Note: It is important to make sure there are no air bubbles surrounding the brain tissue, otherwise the quality of cryopreservation may be compromised. In the case of any air bubbles, remove them with blunt tweezers. Be careful not to damage the tissue during this step.

38. Freeze-embed the brain tissue.
    a. Use long forceps (12″) to grasp the mold with the tissue and gently place the mold into the dry ice/methanol slurry. Make sure that methanol does not directly contact the O.C.T. compound or tissue.
    b. The O.C.T. compound should freeze and turn white, starting from the bottom up and from the sides to the center within 1–2 min.
    c. After the O.C.T. compound fully turns white, transfer the mold on dry ice.

    ⏸ Pause point: The embedded brain tissue block can be stored at −80°C for several months and the procedure can be paused here.

39. Obtain 20-μm brain sections using a cryostat.
    a. Put the O.C.T.-embedded brain tissue inside the chamber of the cryostat and wait until it equilibrates at −20°C.
    b. Mount the tissue block onto a sample holder using a thin layer of O.C.T. compound.
    c. Adjust the angle of the tissue block and the anti-rolling plate so that a flat section can be obtained.
    d. Trim extra tissue until VTA is exposed. Cut 20-μm sections and transfer them to ice-cold 1× PBS solution using a small brush (troubleshooting 6).

Table 1. Dilution ratios of primary and secondary antibodies

Primary antibodies	Dilution ratio	Secondary antibodies	Dilution ratio
Chicken anti-TH	1:1,000	Goat anti-chicken, Alexa Fluor 647	1:250
Mouse anti-TRPV1	1:100	Goat anti-mouse, Alexa Fluor 568	1:200
Rat anti-GFAP	1:500	Goat anti-rat, Alexa Fluor 647	1:200
Rabbit anti-Iba1	1:1,000	Donkey anti-rabbit, Alexa Fluor 594	1:500
Rabbit anti-Cleaved Caspase-3	1:1,000	Donkey anti-rabbit, Alexa Fluor 594	1:500

⚟ **Pause point:** The brain sections can be stored at 4°C for 1 day.

40. Rinse the brain sections 3 times in 1× PBS for 10 min each on a shaker at 450 rpm and 4°C.
41. Perform blocking to prevent non-specific binding.
    a. Prepare the blocking solution by adding 5% normal goat/donkey serum and 0.3% Triton X-100 in 1× PBS.
    b. Place the brain sections in the blocking solution for 1 h at room temperature (20°C–25°C) on a shaker at 150 rpm.

Note: Use serum from the same species in which the secondary antibody is made to make the blocking solution. For example, if the secondary antibody is made in goats, then goat serum should be used in the blocking solution. If secondary antibodies from two or more species are used, use a mixture of serum from each species.

Optional: When performing mouse-on-mouse staining (e.g., mouse is the host species for the primary antibody), an additional blocking step may be adapted to block the endogenous mouse IgG to eliminate non-specific binding. Specifically, incubate the brain sections with AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L) at a dilution ratio of 1:65 in 1× PBS for 1 h at room temperature (20°C–25°C) on a shaker at 150 rpm. Then rinse the brain sections 3 times in 1× PBS for 10 min each on a shaker at 450 rpm and 4°C.

42. During blocking, prepare the primary antibody solution. Dilute primary antibodies in the blocking solution (5% normal goat/donkey serum and 0.3% Triton X-100 in 1× PBS) with appropriate dilution ratios (Table 1).
43. After the blocking finishes, transfer the brain sections from the blocking solution to the primary antibody solution. Incubate the brain sections overnight (12–20 h) at 4°C on a shaker at 150 rpm.
44. The next morning, rinse the brain sections 3 times in 1× PBS with 0.05% Triton X-100 for 10 min each on a shaker at 450 rpm and 4°C.
45. Prepare the fluorescent secondary antibody solution by diluting the antibodies with their appropriate dilution ratios in 1× PBS supplemented with 5% normal goat/donkey serum and 0.1% Triton X-100 (Table 1).

Note: Fluorophores conjugated to secondary antibodies are light-sensitive. Therefore, starting from step 45, caution should be taken to minimize light exposure.

⚠ CRITICAL: Make sure that the fluorophores conjugated to secondary antibodies used for the same section are spectrally separated, otherwise they will not be distinguished in imaging.

46. Transfer the brain sections from the rinsing solution in step 44 to the secondary antibody solution. Incubate the brain sections for 1.5 h at room temperature (20°C–25°C) on a shaker at 150 rpm.
47. Rinse the brain sections 3 times in 1× PBS for 10 min each on a shaker at 450 rpm and 4°C.

48. Mount the brain sections onto glass slides.
    a. Add a few droplets of 1× PBS on a Superfrost Plus glass slide.
    b. Gently pick up a brain section and transfer it to the PBS droplet on the glass slide.
    c. Use Kimwipes to dry the PBS, and the brain section will gradually adhere to the glass slide. Make sure there are no wrinkles or folds in the brain section.
    d. Mount up to 2 brain sections per glass slide.
49. Add 4–5 µL of Prolong Gold Antifade Mountant to the brain section and mount a coverslip on top by slowly lowering the coverslip to touch the mounting solution.

    Note: Make sure to minimize the formation of bubbles near the tissue.

50. Use clear nail polish to seal the edges of the coverslip to prevent the brain section from drying. Allow the nail polish to dry in dark and keep the slides horizontal at room temperature (20°C–25°C) for 24 h.
51. Store the slides in a light-tight container wrapped with aluminum foil before imaging.
52. Perform fluorescence imaging of the brain sections using a confocal microscope.

EXPECTED OUTCOMES

This protocol allows deep-brain neuromodulation in freely moving mice with through-scalp wide-field NIR-II illumination. Example behavioral data from a conditioned place preference test can be found in Figure 5 of (Wu et al., 2022).

LIMITATIONS

Due to the second-level response time of this NIR-II neuromodulation technique (Wu et al., 2022), this protocol is unsuitable for neuromodulation that requires milli-second temporal resolution. It is, however, suitable for neuroscience studies that happen at a longer time scale, such as long-term potentiation in the context of synaptic plasticity. Furthermore, the mechanism of TRPV1 activation requires local heating of the brain, which is controlled to ∼39°C in this protocol. Previous studies have reported that temperature increases inside the brain may suppress neural activities in certain brain regions, thus introducing confounding factors to behavioral experiments (Owen et al., 2019). Therefore, we recommend that users of this protocol always include appropriate controls to confirm that temperature increases at the same level in the brain does not interfere with the anticipated effect of neuromodulation. The resulting effect of elevated temperatures on the molecular, cellular, or synaptic process on certain types of neurons can also be evaluated by *in vitro* studies. In addition, although the NIR-II neuromodulation procedure during the conditioned place preference test is non-invasive, this method still requires invasive stereotaxic injection of AAV and MINDS. Nonetheless, we envision that this remaining level of invasiveness can be further reduced by using systemic delivery of rAAV-PhP.eB virus for TRPV1 transduction (Chan et al., 2017), or creating transgenic animal models that express TRPV1 in certain subgroups of neurons.

TROUBLESHOOTING
Problem 1
The scalp appears to be reddish and dehydrated after the hair removal lotion was removed (step 8b).

Potential solution
The hair removal lotion can cause skin irritation if not removed on time or too much pressure was applied during hair removal. Make sure to remove the lotion within 2 min and be gentle when using the cotton-tipped applicator to remove the lotion. In the case of skin irritation, some vet eye ointment may be applied to the irritated site to slightly alleviate the condition.

Problem 2
Bregma cannot be seen after removal of the scalp (step 10).

Potential solution

It may be challenging to identify bregma immediately after scalp removal when the skull is wet. Gently dry the skull with cotton-tipped applicators and bregma will become more obvious. Additional lighting from a surgical lamp can also be helpful.

Problem 3

Massive bleeding occurs during the drilling process (step 13).

Potential solution

Bleeding may occur when drilling is too deep. Make sure to use the stereoscopic microscope to guide the drilling process and drill slowly. A blower bulb can be used to clean the residue of the skull so that it does not block the view. If massive bleeding occurs, the surgery should be terminated and the animal should not be used for following procedures.

Problem 4

If the needle of the Hamilton syringe was lowered further down after its tip touches the surface of the skull, the sharp needle tip may be bent (step 15).

Potential solution

Be careful when lowering the Hamilton needle. Make sure to use the stereoscopic microscope for better visualization. Bent needle tip can cause various detrimental consequences such as systematic errors in DV positioning and a higher chance in causing bleeding. If the needle tip is bent, replace the tip with a new one.

Problem 5

The brain tissue block still floats on the surface of the sucrose solution after overnight immersion (step 34b).

Potential solution

Gently shake the solution and see if the brain tissue block sinks. If the brain tissue block still floats, continue immersion until it sinks. It is important for the brain tissue block to sink since this behavior indicates that the brain has reached an equilibrium with the sucrose solution and is thus ready for cryopreservation.

Problem 6

Cracks appear in the tissue block and brain sections (step 39d).

Potential solution

The temperature of the tissue might be too low for sectioning, especially if the tissue has been stored at −80°C for extended periods of time. Gently warm the tissue block with a finger or simply leave the tissue block in cryostat for longer time to equilibrate before obtaining the next section.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guosong Hong (guosongh@stanford.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all the data generated during this study.

AUTHOR CONTRIBUTIONS

X.W. and G.H. conceive the idea, develop the methodology, and write the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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