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An Adaptable Angled Stereotactic Approach for Versatile Neuroscience Techniques

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Dear Editor,

Please find attached our re-revised manuscript entitled "An Adaptable Angled Stereotactic Approach for Versatile Neuroscience Techniques" by authors Chelsea L Faber, Miles E. Matsen, Thomas H. Meek, Jordan E. Krull, and Gregory J. Morton. The Editor noted that a few sections of the manuscript overlapped with previously published work, enquired whether the previous publication required explicit permission for reuse and to remove the citation from the abstract.

In response we have revised, reworded and edited sections throughout the Summary, Abstract and Discussion. In addition, we have made additional revisions to Protocol Sections 1, 2, 5 and 6. However, we note that much of the language is written in the preferred style and refers to specific equipment and anatomy and rewriting may change some meaning. The citation in the abstract has been removed and the references renumbered accordingly. In addition, we directly contacted the Editor at Diabetes regarding permission reuse. Given that the Figure in question was only a cartoon, we have now included a revised, updated version of Figure 5 and permission is not required.

We hope you will agree that this revised version of the manuscript is appropriate for publication in JOVE.

Sincerely,

Dr. Gregory J. Morton
Research Professor of Medicine
UW Medicine Diabetes Institute
University of Washington

TITLE:

Adaptable Angled Stereotactic Approach for Versatile Neuroscience Techniques

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KEYWORDS:

CNS, stereotactic surgery, microinjection, optogenetics, chemogenetics, fiber photometry

SUMMARY:

Described here is a stereotactic procedure that can target challenging and difficult-to-reach brain regions (due to spatial limitations) using an angled coronal approach. This protocol is adaptable to both mouse and rat models and can be applied to diverse neuroscientific applications, including cannula implantation and microinjections of viral constructs.

ABSTRACT:

Stereotactic surgery is an essential tool in the modern neuroscience lab. However, the ability to precisely and accurately target difficult-to-reach brain regions still presents a challenge, particularly when targeting brain structures along the midline. These challenges include avoiding of the superior sagittal sinus and third ventricle and the ability to consistently target selective and discrete brain nuclei. In addition, more advanced neuroscience techniques (e.g., optogenetics, fiber photometry, and two-photon imaging) rely on targeted implantation of significant hardware to the brain, and spatial limitations are a common hindrance. Presented here is a modifiable protocol for stereotactic targeting of rodent brain structures using an angled coronal approach. It can be adapted to 1) mouse or rat models, 2) various neuroscience techniques, and 3) multiple brain regions. As a representative example, it includes the calculation of stereotactic coordinates for targeting of the mouse hypothalamic ventromedial nucleus (VMN) for an optogenetic inhibition experiment. This procedure begins with the bilateral microinjection of an adeno-associated virus (AAV) encoding a light-sensitive chloride channel (SwiChR++) to a Cre-dependent mouse model, followed by the angled bilateral implantation of fiberoptic cannulae. Using this approach, findings show that activation of a

subset of VMN neurons is required for intact glucose counterregulatory responses to insulin-induced hypoglycemia.

INTRODUCTION:

Neural control of behavior, feeding, and metabolism involves coordination of highly complex, integrative, and redundant neurocircuits. A driving goal of the neuroscience field is to dissect the relationship between neuronal circuit structure and function. Although classical neuroscience tools (i.e., lesioning, local pharmacological injections, and electrical stimulation) have uncovered vital knowledge regarding the role of specific brain regions that control behavior and metabolism, these tools are limited by their lack of specificity and reversibility[1].

Recent advances in the neuroscience field have greatly improved the ability to interrogate and manipulate circuit function in a cell-type specific manner with high spatiotemporal resolution. Optogenetic[2] and chemogenetic[3] approaches, for instance, allow the rapid and reversible manipulation of activity in genetically defined cell types of freely moving animals. Optogenetics involves the use of light-sensitive ion channels, termed channelrhodopsins, to control neuronal activity. Key to this technique is the gene delivery of channelrhodopsin and a source of light to activate the opsin. A common strategy for gene delivery is through a combination of 1) genetically engineered mice expressing Cre-recombinase in discrete neurons, and 2) Cre-dependent viral vectors encoding channelrhodopsin.

While optogenetics provides an elegant, highly precise means to control neuronal activity, the method is contingent upon successful stereotactic microinjection of the viral vector and fiberoptic placement into a defined brain region. Although stereotactic procedures are commonplace within the modern neuroscience lab (and there are several excellent protocols describing this procedure)[4–6], being able to consistently and reproducibly target discrete brain regions along the midline (i.e., the mediobasal hypothalamus, a brain area critical to the regulation of homeostatic functions[7]) presents additional challenges. These challenges include avoiding of the superior sagittal sinus, third ventricle, and adjacent hypothalamic nuclei. In addition, there are significant spatial limitations to the bilateral implantation of hardware that is required for inhibition studies. With these challenges in mind, this protocol herein presents a modifiable procedure for targeting discrete brain regions via an angled stereotactic approach.

PROTOCOL:

All procedures should be approved in accordance with the National Institutes of Health Guide for the Care and Use of Animals and be approved by both the Institutional Animal Care and Use Committee and Environmental Health and Safety.

1. Calculation of angled coordinates

1.1. Using a coronal brain atlas, mark a right triangle so that the hypotenuse passes through the target region of interest. In the representative example (**Figure 1**), the hypothalamic ventromedial nucleus (VMN) is targeted at a 15° angle from the coronal midline.

NOTE: The placement of the axis of rotation depicted in **Figure 1** (and thus, the length of side C) is arbitrary and can be modified to target any brain region. Although this may seem counterintuitive, later steps in the protocol will adjust the position of the head in the z-axis such that this point aligns with the stereotactic center of rotation (see section 6). However, it is recommended not to exceed a coronal rotation angle of 15° due to physical constraints of the head holder apparatus.

1.2. Establish the desired angle (a) and estimated length of side B and use trigonometry to calculate the length of sides A and C. This step is important for properly positioning the head during rotation.

NOTE: In the example in **Figure 1**, atlas gridlines are used to approximate the length of side B, yielding a length of 7.576 mm. This information is used to calculate the length of side A:

$$tan(15°) = \frac{A}{B} = \frac{A}{7.576\ \text{mm}}$$

$$A = tan(15°) * 7.576\ \text{mm} = 2.03\ \text{mm}$$

In this example, 2.03 mm indicates the R/L distance from the midline at which the fiberoptic cannula enters the brain when the head is rotated by 15°.

1.2.1. Optionally, calculate the length of side C to approximate the D/V coordinate:

$$A^2 + B^2 = C^2$$

$$\sqrt{2.03^2 + 7.576^2} = C = 7.84\ \text{mm}$$

NOTE: 1) The length of the hypotenuse (C) does not represent the depth of injection but will be helpful in determining the D/V coordinate, which may need to be adjusted to accommodate for the increased length vs. side B for straight-in injections. It is therefore recommended to perform test injections to optimize the D/V coordinate. 2) In this example targeting the VMN, two sets of coordinates are obtained: one for the microinjection that is non-angled (A/P = -1.4, R/L = 0.4 at 0°, D/V = -5.7) and one for the angled fiberoptic implantation (A/P = -1.4, R/L = 0.0 at 15°, D/V = -5.4).

2. Preparation of the stereotax for angled procedure

2.1. Confirm that the stereotactic frame and micromanipulator have been calibrated (see Kopf manual for full protocol).

2.2. Place the center height gauge into the socket of the head holder base plate.

2.3. Secure the centering scope in the tool holder, then sight down the scope. Adjust the position of the micromanipulator until the crosshairs are aligned and focused on the gauge crosshairs.

NOTE: During this step, the scope is being positioned into the focal plane of the head holder's center of rotation. Once established, the micromanipulator should not be moved during the remaining steps.

2.4. Place the ear bars into the holders and center them such that the indicator lines on both sides are at 0 (**Figure 3A**).

2.5. Use the medial-lateral and anterior-posterior knobs on the head holder (**Figure 2**) to center-align the ear bars in the x- and y-planes above the crosshair of the center height gauge (**Figure 3A**).

2.6. To align the ear bar position in the z-axis, remove the ear bars from the holder and remove the center height gauge. Replace the ear bars and center them again at 0.

2.7. Sight down the scope. Use the vertical shift knob (**Figure 3B**) and coronal tilt knob, respectively, to lower and rotate the ear bars until the scope crosshairs remain centered between the ear bars throughout coronal rotation.

2.8. The stereotax is now calibrated and ready. Do not make any further adjustments to the position of the head holder.

3. Preparation of materials for injection/implantation

3.1. Ensure that all instruments, surgical tools, and materials are sterilized and placed in a sterile surgical field next to the stereotax.

3.2. Handle and store viral constructs according to their biosafety level and recommended guidelines.

3.3. Draw up the virus into the syringe, taking care to use proper handling practices and personal protective equipment.

4. Anesthesia

4.1. Record the mouse's body weight prior to surgery.

4.2. Deeply anesthetize the mouse using isoflurane.

4.3. Ensure that the mouse is deeply anesthetized by performing a toe pinch test until the flinching response is absent. If the animal continues to show strong reflexes, increase the concentration and/or duration of anesthesia.

4.4. Shave the scalp from just behind the ears to just behind the eyes with a hair clipper.

4.5. Apply eye ointment to each eye to keep moist during surgery.

4.6. Continuously monitor the animal throughout the surgical procedure and provide thermal support, if required.

5. Surgical procedure

5.1. Place the head into the head holder by placing the upper incisors into the gap in the bite bar, making sure that the tongue is below the bite bar.

5.2. Secure the head in the ear bars by gently inserting the ear bars into the external auditory meatus, ensuring that the ear bars are symmetrically placed (typically between three and four for an adult mouse). This step is critical to ensure the head is stable and centered for rotation.

5.3. Sterilize the shaved incision area with three alternating scrubs of betadine and alcohol swabs.

5.4. Expose the skull by making an incision along the sagittal midline of the scalp. Gently scrape the surface of the skull to remove any fascia and expose the sutures.

NOTE: If suture lines are difficult to visualize, hydrogen peroxide can be applied to the skull using a sterile cotton-tipped applicator to improve suture visualization.

5.5. Place the centering scope into the holder and center the crosshairs on bregma (**Figure 4**, left panel). Zero the micromanipulator.

5.6. Move the crosshairs caudally to lambda, noting the bregma-lambda (B-L) distance.

NOTE: If the suture lines do not follow a straight line along the midline, it is recommended to establish the midline using the “line of best fit” through both bregma and lambda. However, if the above steps are followed, the initial placement of the scope reticle should be halfway between the ear bars and closely approximate the B-L midline suture.

5.7. If the B-L distance is significantly less or greater than 4.21 mm, incrementally adjust the assigned bregma to obtain a B-L distance of 4.21 mm ± 0.2 mm.

5.8. Replace the centering scope with the alignment indicator. Place the probes on lambda and bregma and adjust the dorsal tilt knob on the head holder to level in the sagittal plane (nose facing up or down), then use the centering scope to reassign bregma.

5.9. Use the alignment indicator to level in the coronal plane using the coronal tilt knob. Measure at multiple points throughout the rostral/caudal axis to account for surface deformations in the skull.

5.10. Note the position on the dial of the coronal tilt knob, as this is the 0° rotation position.

6. Aligning the central axes of rotation for angled coordinates

6.1. Secure the centering scope in the tool holder and position the micromanipulator to the calculated coordinate from section 1. Note that the R/L coordinate for the angled implantation corresponds to the length of side A.

6.1.1. In the example in **Figure 1**, the angled coordinates for fiberoptic placement targeting the VMN are (A/P = -1.4, R/L = [2.03] at 0° coronal rotation, [0.00] at 15° coronal rotation, D/V = -5.4).

6.2. Sighting down the scope and mark this coordinate (R/L 2.03 mm from the midline per the VMN example; **Figure 4**, middle panel). This mark represents the point at which the cannula will enter the brain once the head is rotated.

6.3. Reposition the micromanipulator over the midline (R/L = 0.00). Use the coronal tilt knob to rotate the head to the angle calculated in section 1.

6.3.1. If the scope crosshairs already line up with the mark, proceed to section 7.

6.3.2. If the scope crosshairs do not line up with the reference mark, adjust the head position in the z-axis using the vertical shift knob (**Figure 2**) until the crosshairs line up as close as possible to the mark.

6.4. Rotate the head back to the 0° coronal position. If the vertical shift was adjusted in step 6.3, reassign bregma using the centering scope.

6.5. Repeat steps 6.3 and 6.4 until the crosshairs consistently hit the reference mark when the head is rotated (**Figure 4C**).

6.6. At this point, the arbitrary point of rotation established in section 1 should now be aligned with the stereotactic center of rotation.

7. Microinjection

7.1. Place the stereotactic drill in the holder and maneuver the micromanipulator to the first injection coordinate.

7.1.1. Per the example for targeting the VMN, drill at A/P = -1.4 and R/L = 0.4 while the head is level.

7.2. Lower the drill until the bit is just above the skull. Turn on the drill, and gently lower until the bit has just drilled through the skull (not the dura).

7.3. Repeat for the contralateral injection site.

7.4. Gently poke through the dura mater using the tip of a sterile 0.5 mL insulin syringe that is bent to 90°.

NOTE: If bleeding occurs, apply pressure with a sterile cotton-tipped applicator and clean with sterile water until the bleeding has stopped.

7.5. When ready to inject, carefully place a filled Hamilton syringe into the stereotactic holder.

NOTE: The coordinates on the micromanipulator no longer apply after switching to a new tool. Use the center of the burr hole as the new target for injection.

7.6. Carefully position the needle above the burr hole.

7.7. Lower the needle until it slightly touches the dura within the center of the burr hole. CRITICAL: Zero the micromanipulator only in the z-axis, such that the coordinates on the micromanipulator for the stereotactic centering scope and drill are maintained.

7.8. Slowly lower the needle into the brain, watching closely to ensure that the needle does not deflect on the edge of the burr hole. Continue to lower until 0.05 mm ventral to the D/V injection coordinate and wait 1 min. This extra step creates a small “pocket” to minimize viral backflow on needle removal.

7.9. Slowly raise the needle to the D/V coordinate and start the injection.

NOTE: Flow rate and volume will vary depending on target region and experimental design. For optogenetic silencing of VMN neurons, sufficient coverage is desired, so 200 nL of virus is injected at a rate of 1 nL/s.

7.10. Following microinjection, wait 10 min at the injection site to minimize efflux of virus during withdrawal.

7.11. Slowly withdraw the micropipette from the brain at an approximate rate of 1 mm/min.

7.12. Once the needle is clear of the skull, eject a small volume of virus to ensure the needle has not clogged with blood or tissue. Use a sterile cotton-tipped applicator to remove the virus before continuing.

7.13. Repeat steps 7.6–7.12 for the contralateral side.

7.14. Seal the microinjection burr holes with bone wax to improve healing (**Figure 5B**).

8. Fiberoptic implantation

NOTE: After viral injection, bilateral fiberoptic cannulas are implanted at the calculated angle per section 1. Note that these coordinates should already be marked on the skull from section 6.

8.1. Repeat steps 7.1 –7.4 for the angled coordinates.

8.2. Return the head to the level 0° position.

8.3. Next, use the hand drill to produce four additional holes for the bone screws: two should be placed anteriorly and two posteriorly (**Figure 5A**). These will serve as anchors to affix the fiberoptics to the skull (**Figure 5D**).

NOTE: Make sure to space the holes far enough away from the angled coordinate burr holes to accommodate the ferrule portion of the fiberoptic that sits above the skull.

8.4. As gently as possible, use the small flathead screwdriver to set the bone screws such that they sit firmly in the skull but do not penetrate the brain.

8.5. Clamp a fiberoptic cannula into the cannula holder and place it in the stereotactic holder.

8.6. Rotate the head to the calculated angle, noting again that the coordinates on the micromanipulator do not apply to the new tool. Use the center of the angled burr holes as the implantation target.

8.7. Lower the fiberoptic until it just touches the dura within the center of the burr hole (**Figure 5C**). Zero the micromanipulator in the z-axis, then slowly slower the fiberoptic to the D/V angled coordinate (-5.4 per the VMN example).

8.8. Use cyanoacrylate gel to connect the fiberoptic ferrule to the ipsilateral anchor screws, then apply an accelerant with a micropipette tip (**Figure 5D**).

8.9. Once the cyanoacrylate gel has completely hardened, gently loosen the cannula holder and raise until clear of the fiberoptic ferrule.

8.10. Repeat steps 8.5–8.9 for the contralateral angled coordinate, then level the head. For extra security, make an additional connection between the two angled fiberoptic cannulas with the cyanoacrylate gel and accelerant (**Figure 5D**).

8.11. Prepare a small, relatively thin amount of dental cement. Apply to the surface of the skull, making sure to thoroughly cover the anchor screws and base of the fiberoptic cannulas. Leave enough of the ferrule clean for subsequent mating with the fiberoptic patch cables.

8.12. Once the cement is completed dry, remove the mouse from the stereotactic apparatus.

8.13. Inject the mouse subcutaneously with analgesic (buprenorphine: 0.5mg/kg)

8.14. Place mouse in a recovery cage with thermal support. Allow it to recover and transfer to the home cage once it appears alert, mobile, and is grooming.

9. Post-surgical care

9.1. Monitor animals daily for 3 days post-operatively for behavior, posture, activity, and grooming, and keep records of food intake and body weight.

9.2. If animals exhibit any general indicators of pain or poor health, consult with veterinarian services.

9.3. Allow mice at least 2 weeks for recovery and for viral expression before starting behavioral studies.

10. Optogenetics

10.1. For the performance of optogenetics studies, refer to Sidor et al.[8].

10.2. Validate viral expression and fiber placement at the completion of studies.

REPRESENTATIVE RESULTS:

This protocol describes a surgical procedure for performing optogenetics studies to interrogate the role of hypothalamic VMN neurons in glycemic control[9]. First utilized was a standard (non-angled) stereotactic approach for the bilateral microinjection of an inhibitory channelrhodopsin virus to the VMN. While an angled approach would also be suitable, the standard (non-angled) approach was selected because it is sufficient to target the brain region of interest and is an easy, reliable and consistent approach. However, given the VMN's proximity to the midline, space constraints did not permit the non-angled implantation of bilateral fiberoptics, necessitating the development of a surgical strategy for precisely implanting fiberoptics at an angle (**Figure 6**).

Using this surgical strategy, we microinjected a Cre-dependent AAV expressing a modified channelrhodopsin anion-conducting channel fused with the fluorescent reporter, referred to as a "SwiChR++" virus[10], bilaterally to the VMN of Nos1-cre mice. This was followed by implantation of an optic fiber dorsolateral to each injection site at a 15° angle from the midline. As expected, viral expression was restricted to the VMN and not detected in other brain areas.

FIGURE LEGENDS:

Figure 1: Representative example of calculating angled coordinates targeting the hypothalamic ventromedial nucleus. Angles and line segments are not drawn to scale. **(A)** This length should be calculated using basic trigonometry. In this example, A = 2.03 mm. **(B)** Estimated length based upon assignment of arbitrary axis of rotation. In this example, B = 7.576 mm. **(C)** Calculated hypotenuse. It should be noted that the depth of fiberoptic/needle insertion depends upon the desired proximity to the target region, which requires optimization. This figure has been modified from Faber et al. 2019[11].

Figure 2: Adjustment knobs for the stereotactic head holder apparatus. This figure has been modified from Faber et al. 2019[11].

Figure 3: Aligning the head holder center of rotation. (A) Positioning the ear bars. **(B)** Sighting down the scope during 0° level coronal rotation (left), during 15° rotation before adjusting the vertical shift, and the center of rotation is misaligned (middle), and during 15° rotation after adjusting the vertical shift, and the center of rotation is properly aligned (right). This figure has been modified from Faber et al. 2019[11].

Figure 4: Assigning bregma and aligning the animal head with central axes of rotation. (A) Representative image indicating typical bregma placement. **(B)** Drawing a reference mark while head is level, before alignment. **(C)** Properly aligned axis of rotation, after adjusting the vertical shift and readjusting bregma.

Figure 5: Fiberoptic implantation procedure. (A) Centering scope view of pilot holes for microinjection (m), fiberoptic (f), and anchor screws (*). **(B)** Centering scope view of implanted anchor screws, and bone wax covered microinjection drill holes. **(C)** Positioning the fiberoptic into place during angled implantation. **(D)** Representative bilateral angled fiberoptic placement. Dotted black arrows indicate areas in which super glue is used to anchor the fiberoptic to the anchor screws and ipsilateral fiberoptic.

Figure 6: Representative results for bilateral targeting of the ventromedial hypothalamus. (A) Schematic representing bilateral microinjection and angled fiberoptic strategy for targeting the VMN. **(B)** Representative image showing bilateral expression of SwiChR-GFP and tissue damage from angled fiberoptic tracts. 3V = third ventricle, ARC = arcuate nucleus, and VMN = ventromedial nucleus.

DISCUSSION:

Recent advances in neuroscience have supported advanced insight and understanding into the activity and function of brain neurocircuits. This includes the application of optogenetic and chemogenetic technologies to activate or silence discrete neuronal populations and their projection sites in vivo. More recently, this has included the development of genetically encoded calcium indicators (e.g., GCaMP, RCaMP) and other fluorometric biosensors (e.g., dopamine, norepinephrine) for in vivo recording of neuronal activity in a defined cell type in freely moving animals. However, effective employment of these technologies relies upon successful stereotactic surgery to target the region of interest. While there are several established protocols describing these methods, which are suitable for targeting many brain regions, targeting deep brain regions along the midline represents significant additional challenges. Demonstrated here is a detailed surgical technique for targeting discrete brain regions via an angled stereotactic approach. Importantly, this technique can be adapted and applied to a diverse range of neuroscience techniques (i.e., optogenetics, chemogenetics, and fiber photometry approaches).

Using this approach, it is shown that acute optogenetic silencing of VMN neurons expressing neuronal nitric oxide synthase (VMN^{NOS1} neurons) blunts glucagon responses to insulin-induced hypoglycemia in mice[9]. Using a slightly modified approach, it is further demonstrated that unilateral activation of VMN^{NOS1} neurons 1) elicits robust hyperglycemia that is driven by counterregulatory responses that are normally reserved for the response to hypoglycemia, and 2) elicits defensive immobility behavior. Furthermore, these behavioral and metabolic responses involve neuronal projections to distinct brain areas. Specifically, the activation of VMN^{NOS1} neurons projecting to the anterior bed nucleus of the stria terminalis are involved in glycemic responses, whereas VMN^{NOS1} neurons projecting to the periaqueductal gray are linked to fear-induced behavior responses[9].

It should be noted that the protocol is highly specific to the Kopf Model 1900 stereotax and accompanying accessories. While this system enables precise, reproducible implantation as well as microinjection to discrete brain regions (with a common centerline position across multiple tools), the strategy and approach can be adapted to suit other stereotaxic frames. Specifically, instead of rotating the head to perform angled microinjections and implantations, an alternative approach is to utilize the same principles and rotate the dorsal-ventral manipulator instead (see Correia et al.[12]).

As with any new method, it is critical for individuals to optimize the technique to improve an experiment's reliability, consistency, and accuracy. In addition, it is important to include the necessary appropriate controls for proper analysis and interpretation of data. These include the use of Cre-negative littermate controls, viral reporter controls (i.e., AAV-GFP), verification of light-dependent neuronal firing modulation using electrophysiology, and (upon study completion) the validation of viral targeting and fiberoptic placement in the region of interest. It is recommended to refer to the publication by Cardozo and Lammel[13] for a detailed review of technical considerations and suggested controls.

In summary, the introduction of more advanced and precise neuroscience techniques has supported a significant advancement and understanding of the role of the brain in behavior, cognition, and physiology, and these advancements may lead to potential therapies for CNS-related disorders.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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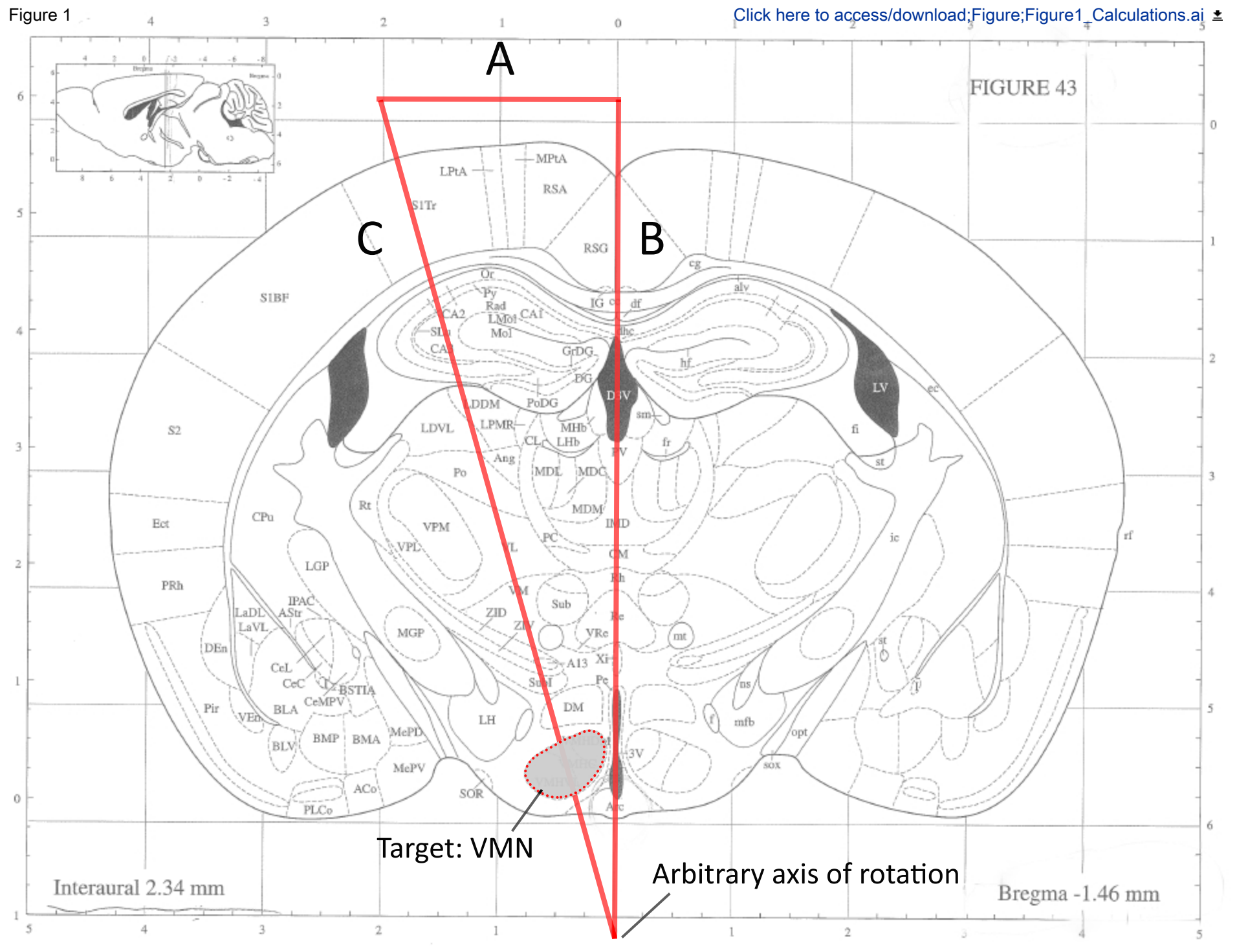


Figure 2

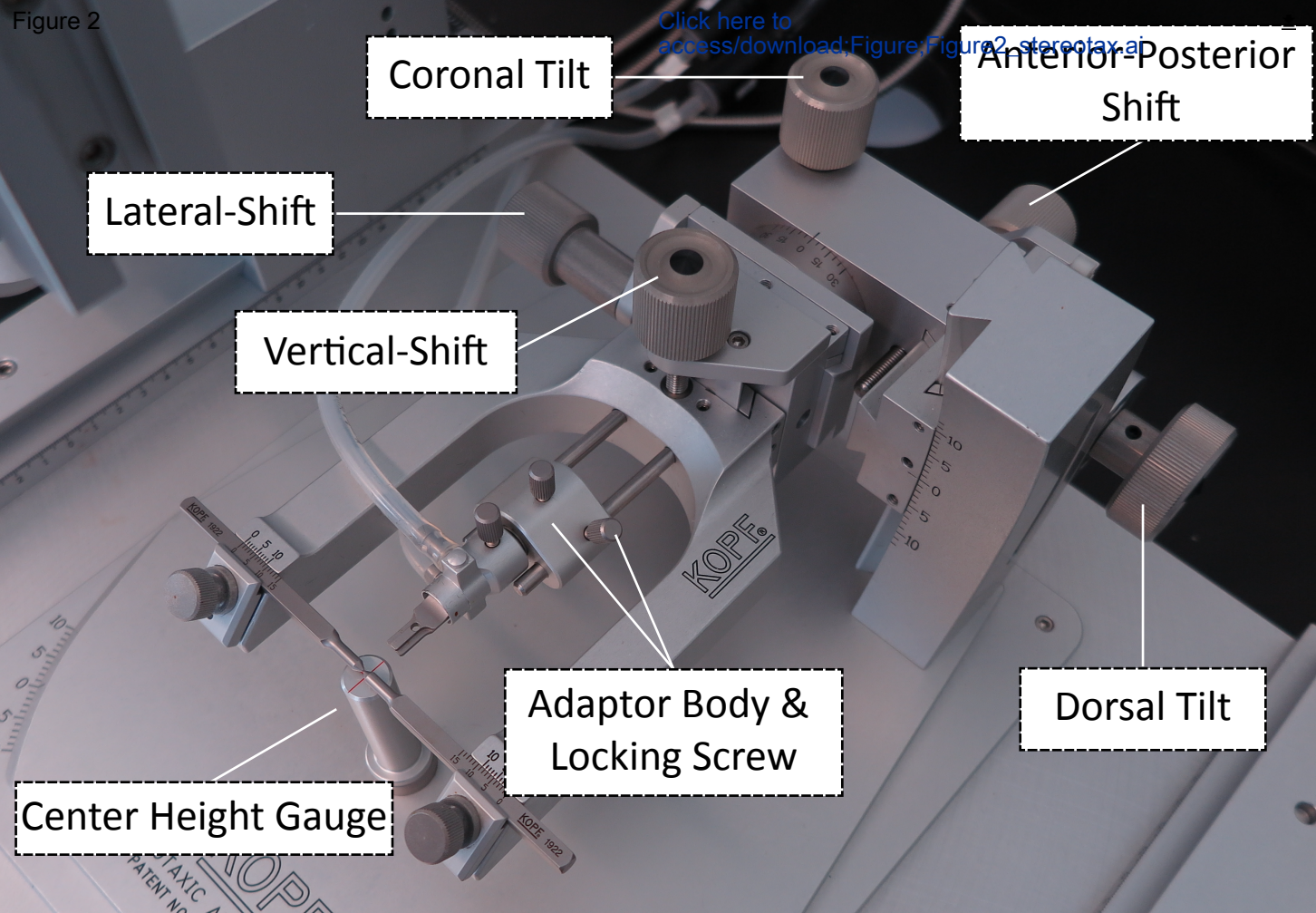
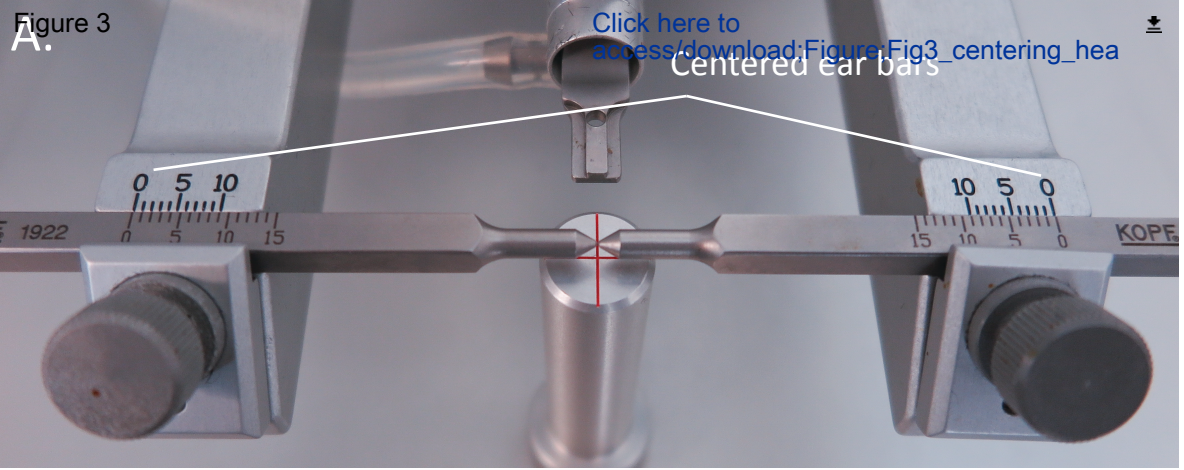
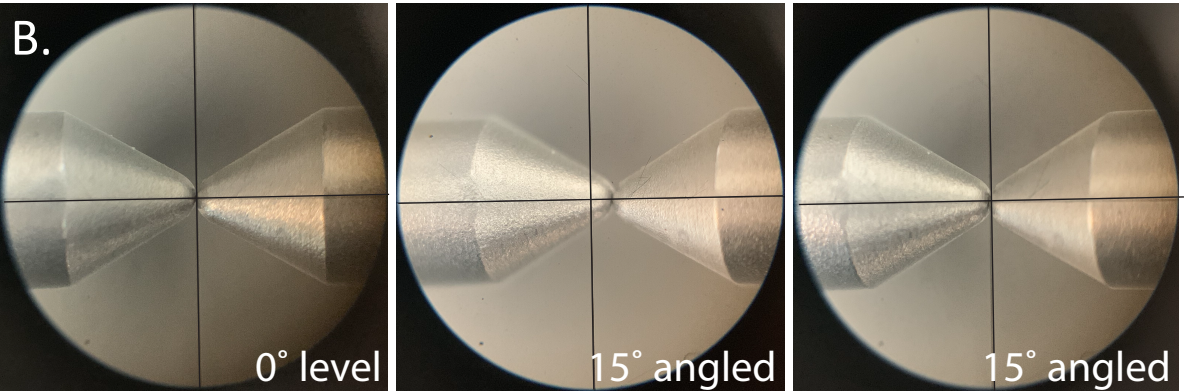
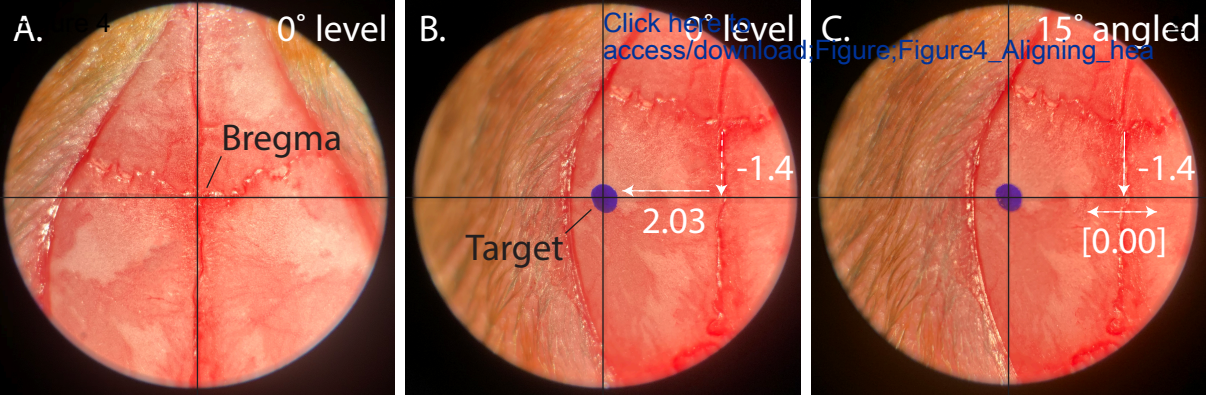


Figure 3






A.
0° level
Bregma
B.
0° level
-1.4
2.03
Target
C.
15° angled
-1.4
[0.00]

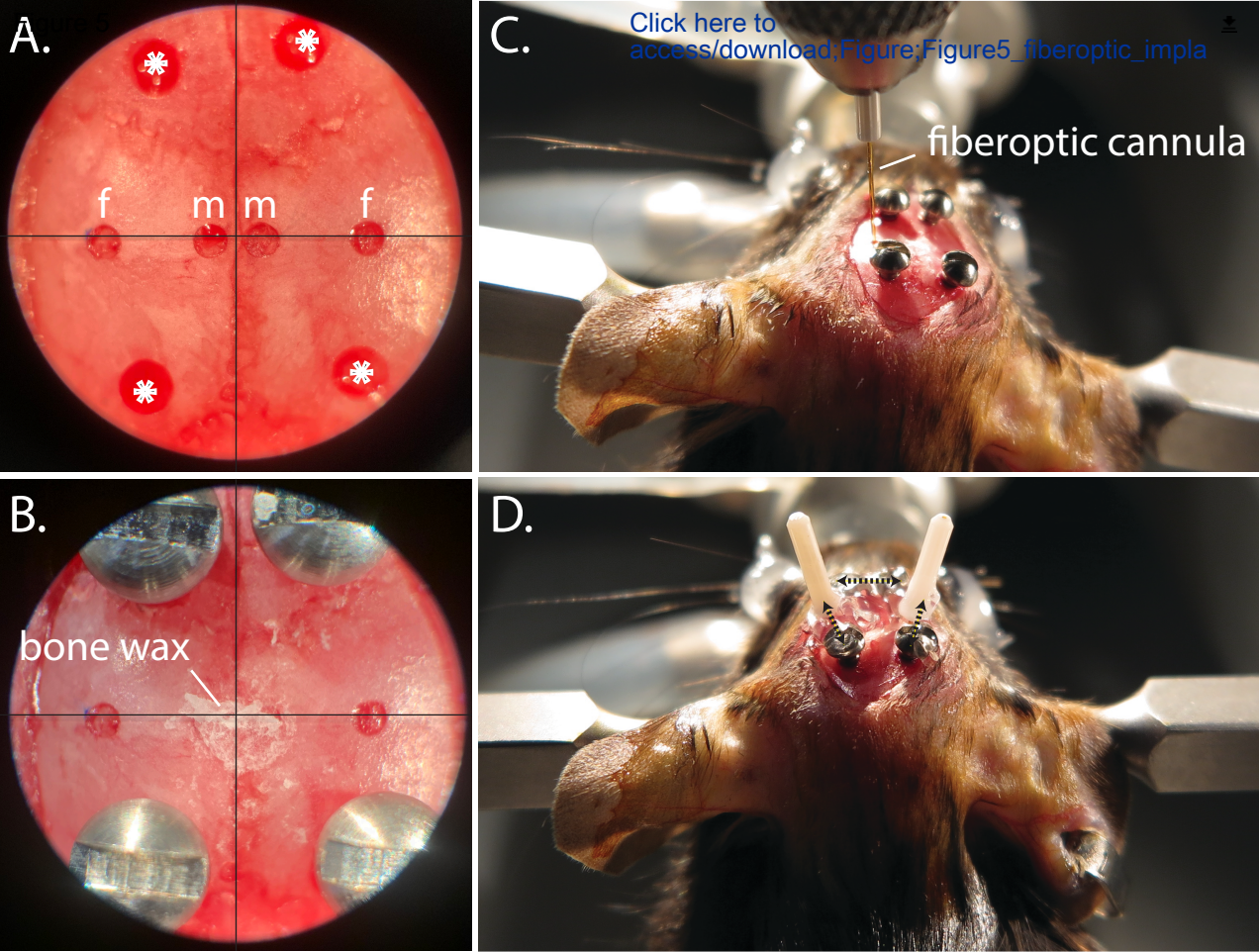

A.
f
m
m
f
B.
bone wax
C.
Click here to
access/download;Figure;Figure5_fiberoptic_impla
fiberoptic cannula
D.

Figure 6

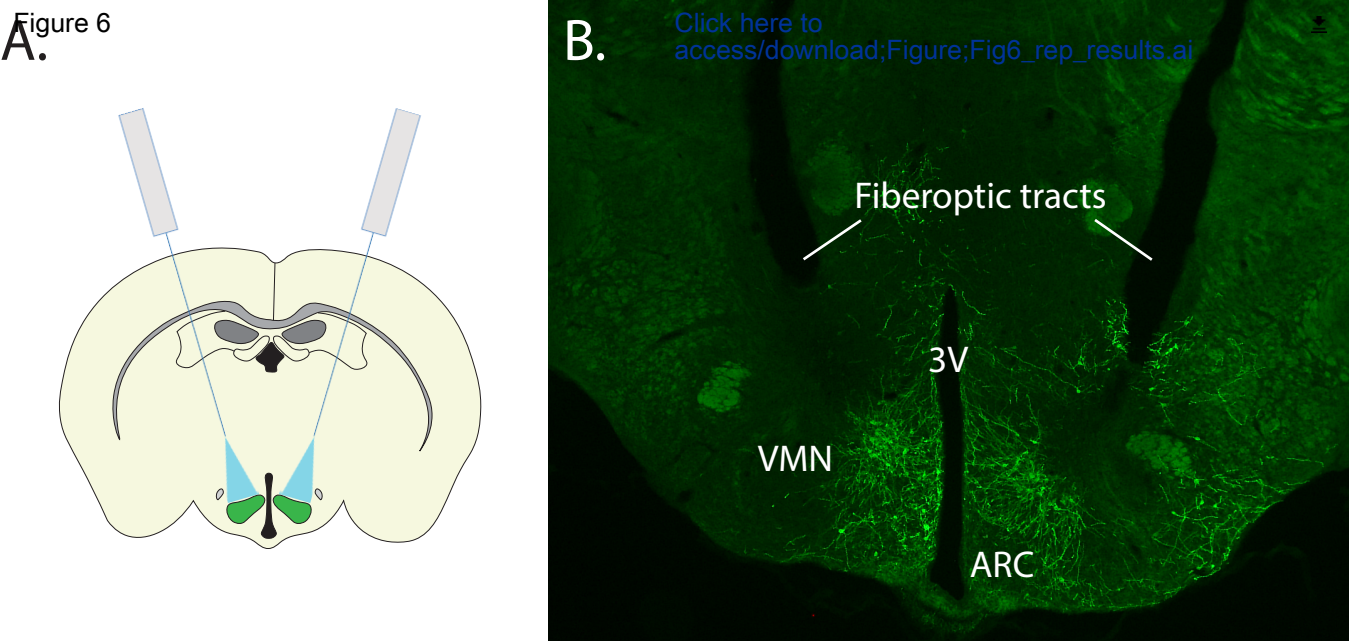

A.
B.
Click here to access/download;Figure;Fig6_rep_results.ai
Fiberoptic tracts
3V
VMN
ARC

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Fiberoptic Cannulae	Doric Lenses	MFC_200/230-0.57_###_MF1.25_FLT
Kopf Model 1900 Stereotaxic Alignment System	Kopf	Model 1900
Kopf Model 1900-51 Center Height Gauge	Kopf	Model 1900-51
Kopf Model 1905 Alignment Indicator	Kopf	Model 1905
Kopf Model 1911 Stereotaxic Drill	Kopf	Model 1911
Kopf Model 1915 Centering Scope	Kopf	Model 1915
Kopf Model 1922 60-Degree Non-Rupture Ear Bars	Kopf	Model 1922
Kopf Model 1923-B Mouse Gas Anesthesia Head Holder	Kopf	Model 1923-B
Kopf Model 1940 Micro Manipulator	Kopf	Model 1940
Micro4 Microinjection System	World Precision Instruments	--
Mouse bone screws	Plastics One	00-96 X 1/16
Stereotaxic Cannula Holder, 1.25mm ferrule	Thor Labs	XCL
Surgical Drill	Cell Point Scientific	Ideal Micro Drill

Comments/Description
Customizable

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please remove references from the Abstract and renumber accordingly.

Protocol:
1. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Figures:
1. If applicable, please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

References:
1. Please include at least 10 references.
2. Please do not abbreviate journal titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We thank each of the Reviewers for their positive comments and their constructive feedback. In response, we have made extensive revisions that provide additional clarity and detail and further highlighted the advantages and practicalities of this technique. We believe these revisions have significantly strengthened the manuscript and a point-by-point response to each of the Reviewers comments is outlined below.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript presented by Morton et al describes a customizable protocol for targeting brain structures using an angled stereotaxic approach which is suitable for either mouse or rat and can be modified for diverse experimental approaches and brain regions. In advance, the manuscript is well written and will be very useful for the readers that will need to use the optogenetics or chemogenetics approaches. I have high expectations to watch the video.

Minor Concerns:
I have just three minor suggestions.
1. To measure the "A" distance on the rodent's skull, what should the researcher do if the midline fissure is not straight line? Follow the bregma coordinate or the bone fissure? I encourage the authors to discuss this issue in session 6.

The Reviewer raises an important issue regarding the challenges associated with the identification of bregma given that markings are not always symmetrical. If the midline fissure is not a straight line,

we recommend taking a “line of best fit.” We have incorporated this additional Note in the revised Section 5.4.

2. According to Figure 5A, in Figure 5 legends, microinjection and fiberoptic abbreviations should be "m" and "f", respectively, instead of "MI" and "F".

We thank the Reviewer for catching this error. The Figure Legend has been revised accordingly.

3. In the "Table of Materials" session, the authors should include the specification of anchor screws and fiberoptics for mice.

These materials have been added to the Table of Materials.

Reviewer #2:
This describes a method in neuroscience for stereotaxic injection/implantation that has been the subject of multiple methods papers. The authors report a method for making injections off the vertical axis by tilting the animal's head in a specific Kopf frame. The instructions are so specific to that frame that it is in essence a manufacturers user manual for this approach. It is unlikely that many labs have this particular frame and those that do could simply read the user manual. There is nothing about what the authors describe that is anything other than pretty standard. As such we are am not convinced this merits publication as a method (several similar methods descriptions have already been published in JOVE and in other publications - listed below). The authors also do not provide any evidence that this provides better results than simply angling the manipulator for the injection / implant.

https://www.jove.com/video/59534/
https://www.jove.com/video/53783/
https://www.jove.com/video/59465/
https://www.jove.com/video/52653/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5633075
https://www.nature.com/articles/srep38058

We concur with the reviewer that there are already several excellent neuroscience protocols for both injection and implantation. These are an excellent resource that are widely applicable but are limited with the challenges associated with targeting deep brain structures that are positioned close to the midline. This is particularly the case for implantation of necessary hardware for fiber photometry and optogenetic studies which is constrained by limited space.

We acknowledge that the detailed protocol is highly specific to the Kopf stereotactic frame, a concern also raised by several additional Reviewers. To address this, we have revised the Discussion to include suggestions for alternate strategies accommodating different stereotactic frames, and to clarify the advantage of this particular method.

Specifically, the revised Introduction states “Although stereotaxic stereotactic procedures are common place within the modern neuroscience lab and there are several excellent protocols describing this procedure (Richevaux et al; J Vis Exp 2019, Fricano-Kugler et al; J Vis Exp 2016, McSweeney and Mao, J Vis Exp 2015) being able to consistently, reproducibly and reliably target discrete brain regions along the midline, such as the mediobasal hypothalamus, a brain area critical in the regulation of homeostatic functions (Lowell BB; NEJM 2019) represents significant additional challenges. These challenges include avoiding the superior sagittal sinus, the third ventricle and hitting adjacent hypothalamic nuclei. In addition, there are significant spatial limitations for the

bilateral implantation of hardware that is required for inhibition studies. With these challenges in mind, we herein present a modifiable procedure for targeting discrete brain regions via an angled stereotactic approach.

In the revised Discussion, we state “We note that the protocol described is highly specific to the Kopf Model 1900 stereotax and its accompanying accessories. While this system enables precise, reproducible implantation and/or microinjection to discrete brain regions, the strategy and approach can be adapted to suit other stereotaxic frames. Specifically, instead of rotating the head to perform angled microinjections and implantations, an alternative approach is to utilize the same principles and rotate the dorsal-ventral manipulator instead.”

Reviewer #3:
Manuscript Summary:
The manuscript by Morton and colleagues describes a method for angled stereotaxic implantation of optical fibers into the rodent brain. This is a valuable technique that addresses the limitations of standard stereotaxic delivery approaches and could be widely useful to the neuroscience community. Overall, their procedures are well described and provide a good description of this methodology. I offer the following suggestions to improve the accessibility of the manuscript.

1) Calculated angled coordinates: The authors describe the trigonometry used to calculate side A of the triangle, but do not describe how they calculated the DV coordinate (a shorter version of side C). Although most readers will be adept at math and can figure this out, it would be useful for them to describe this.

Additional steps have been added to Section 1 to clarify calculation of the DV coordinate.

2) It would be helpful to have a description of the function for the knobs on the stereotaxic system in Figure 2, as it is challenging to decipher what is happening in the step 2 section. Perhaps this could be in the protocol or in the figure legend.

We elected not to include a detailed description of the function for the knobs on the stereotaxic system in Figure 2 given that this information is readily available in the Kopf User Manual to which we direct readers attention (see Section 2.1). However, in the accompanying video, we intend to give an overview of the stereotaxic setup and describe each of the individual components.

3) The authors could comment on why they use the standard angle for viral delivery and angled targeting only for implantation. Why not do both with angled targeting?

The angled targeting approach can be successfully utilized for both viral delivery and for implantation. However, the decision to use a standard or an angled approach depends on the brain region to be targeted. In the current example, the standard approach was an acceptable method to target the brain region of interest and was selected given it is reliable, consistent and easier. In contrast however, there are generally more limitations for implants, particularly when targeting brain regions along the midline given their size and shape. Thus, in order to target the implant to the hypothalamic ventromedial nucleus, an angled approach was required. We have added additional language in the revised text (see Representative Results) to describe this decision-making process and stated that either strategy is suitable and acceptable.

4) The methodology described is highly specific to the Kopf 1900 stereotaxic system. However, it seems plausible that the approach could be adapted to other stereotaxic systems, by rotation of the dorsal-ventral manipulator rather than the rotation of the mouse head. It would be nice to add some

discussion for how the technique might be adopted to other setups.

This is an important point that was raised by several Reviewers. To expand the versatility of this protocol, it can be adapted by rotation of the dorsal-ventral manipulator rather than the rotation of the mouse head. We have highlighted and emphasized this important point throughout the revised manuscript and described it as an alternative in the Revised Summary and Discussion sections.

Reviewer #4:

Manuscript Summary:

The authors present an adaptable protocol for targeting discrete brain structures using an angled stereotaxic approach. This protocol would be of substantial interest to the neuroscience community as a solution for manipulating structures that are positioned near the sagittal midline and circumnavigating brain regions immediately dorsal to targeted structures. Although angled stereotaxic approaches are already implemented by members of the neuroscience community, this protocol addresses the lack of uniform, reproducible practice across groups and offers a systematic and flexible methodology for the calculation of precise coordinates for a wide range of subcortical structures.

Major Concerns:

Generally, the protocol is thoroughly and clearly written, and I think that the steps listed would achieve the desirable outcome. One general concern is that the protocol can be a little confusing without a clear visual guide when there are many references to specific components (eg. part 2, part 5), but this could be improved with the provision of additional figures and will likely be further clarified with the video.

We found it difficult to include all the relevant details and specific components of the scope and stereotaxic apparatus in a single, or series of Figures. However, given the importance with being familiar with both the orientation and the components of the set-up, additional attention will be proceeded with the accompanying vides which should provide further clarity. Please also refer to our response to Reviewer #3, Comment 2.

Minor Concerns:

I have enclosed some specific comments with the hopes of improving the manuscript:

Lines 112-115: The authors touch on the issue of reproducibility in lines 108-10, but I recommend stating this explicitly in this closing statements as a key strength of their protocol.

We thank the reviewer for this suggestion, and we have revised the Discussion accordingly.

Line 150-152: To clarify how the lengths/coordinates presented in Section 1.2.1 are derived, state explicitly here that the length of B & C are calculated based on gridlines.

As indicated in the original manuscript, the length of B is calculated on gridlines, however, due to a similar comment by Reviewer #3, we have clarified the calculation of side C the DV coordinate (see revised Section 1).

Line 173: It would be useful to expand Fig 2 to illustrate the components of the frame that are manipulated in 2.2-2.4.

The components adjusted in steps 2.2-2.4 are illustrated in Figure 3 of the original manuscript.

Line 192: Consistency check for component names - line 173 refers to 'Center Height Gauge'.

We thank the reviewer for pointing out this inconsistency. The text has been revised accordingly.

Line 198: One reference to Figure 3B is sufficient.

The text has been revised accordingly.

Line 251: Label panels in Fig 4 as A, B, C for clearer reference.

The Figure and Figure Legend have been revised accordingly.

Line 266-267: 'Make sure to note…' should be included as a separate step or moved to 5.8.

We disagree that this line of the text should be moved, as changes may continue to be made to the coronal tilt after reassigning bregma. Therefore, the position of the dial should be noted last, after all adjustments have been made.

Line 374: Sealing with bone wax is included twice in the protocol (see line 355).

We thank the reviewer for catching this redundant comment, which has been removed.

Line 437-447: The authors reference the published work that used this technique, but the reader would appreciate a sentence or two in this section which summarises their findings alongside their description of the methodology to provide richer functional context.

We thank the Reviewer for this suggestion. In response, we have added additional statements that summarize the findings of the referenced study.

Line 504-505: Include an example(s) of what would constitute an appropriate control within the present experimental design.

Inclusion of the appropriate controls is critically important in the analysis and interpretation of data. This includes use of Cre-negative littermate controls, viral reporter controls (i.e. AAV-GFP), verification of light-dependent neuronal firing using electrophysiology and, upon study completion validation of viral targeting and fiberoptic placement in the region of interest. For more detailed information on appropriate controls in the field, we direct readers to previous publications.

Reviewer #5:
Manuscript Summary:
In this paper, Faber et al. describe a surgical strategy for targeting specific brain structures with an angled stereotaxic approach. The strategy allows microinjections of reagents and implantation of devices into specific brain regions. Compared to the standard stereotaxic technique, the angled stereotaxic approach may permit targeting deep brain nuclei without damage to the overlying superior sagittal sinus or overlying brain structures.

In General, the protocol is well described. However, some issues need to be addressed.

Major concerns:
(1) The stereotaxic apparatus used by the author needs to adjust several parameters, but the traditional stereotaxic apparatus only needs to adjust the angle of the lateral holder arm for

microinjection and implantation. What are the advantages of the stereotaxic apparatus used by the author over the traditional stereotaxic apparatus?

This is an important point raised by additional Reviewers. We have endeavored to highlight and feature the advantages of this approach relative to use of the traditional stereotaxic apparatus. Please see our Response to Reviewer #2 for a detailed description. In response, we have made several revisions and additions throughout the manuscript.

(2) Line 447: The authors claimed that "As expected, viral expression was restricted to the VMN and not detected in other brain areas." However, the data seem not to support the target specificity. In Figure 6B, in addition to the targeted VMN, the GFP signals also appear in the left ARC region, despite that Nos1-Cre driver and Cre-dependent AAV virus were used.

We agree that there is some leakage of virus outside the VMN, however, restriction is largely limited to the VMN. A key limitation to VMN-specific targeting is the expression of the Nos1-cre driver in regions outside the target region, including ARC, DMH, and LHA. Therefore, specificity of SwiChR-YFP expression is constrained by viral spread, and not by the cre-line.

Minor concerns:
(1) Line 165: D/V: -5.7 and line 166 D/V: -5.4. How to get D/V: -5.4 at 15。 from D/V: -5.7 at 0。?

This point was raised by Reviewer #3, as well. We therefore have added clarifying information to section 1 (see above).

(2) Line 177: Please provide a photo containing the scope and stereotaxic apparatus to demonstrate the spatial arrangement of the scope and stereotaxic apparatus.

It is difficult to obtain a photo that contains the full spatial arrangement of the scope and stereotaxic apparatus without sufficient detail in a single, or series of Figures. However, we believe this is still an important point and will give additional attention to this detail which should be further clarified in the accompanying video. Please also refer to our response to Reviewer #3, Comment 2.

(3) Line 173 and 188: The Centering Height Gauge should be indicated in Figure 2.

Per the reviewer's suggestion, the center height gauge has been labeled in revised Figure 2.

(4) Line 214: Keeping the animal on the warm pad during the entire surgical procedure.

Depending on the length of the surgery, thermal support may be recommended (see step 4.6).

(5) Line 250: What is the difference between the Centering Scope and the Stereotaxic Scope?

We apologize for the inconsistent terminology. The text has been revised accordingly

(6) Step 8.4 (line 374): The procedure to apply bone wax to the holes drilled for microinjection only is already done in Step 7.14 (line 355).

We thank the Reviewer for catching this redundant comment, which has been removed.

(7) Step 8.6 (line 378): Please describe the details of how to rotate the animal head and how to center the burr hole.

We have not described these details in the text but will ensure that we explain this procedure in detail in the accompanying video.

(8) Figure 3B: Mark the angled degree in each photo. This set of images does not show how to turn 0。to 15。 and adjust to the center.

We thank the Reviewer for this suggestion to improve clarity. The figure has been revised accordingly.

(9) Figure 4: The 0。level photo (middle) and 15。level photo (right) are the same. What is the difference between these two photos?

The middle and right panels are not the same; the angled position of the head is indicated in the top left of each panel.

(10) Figure 5E: The photo is out of focus.

We thank the Reviewer for noting this. We subsequently determined that Figure 5E does not add further additional information relative to Figure 5D and has been removed.

Reviewer #6:

Manuscript Summary:

The current submission describes a protocol for angled placement of fiber optics to target deep mid-line structures in the rodent brain. The authors give clear rationale for the importance and most steps are clear and easy to follow.

Major Concerns:

The steps for calculating coordinates are not entirely clear and would benefit from additional breaking down of the steps (especially for readers who have not had trigonometry for quite some time). The sagittal aspect of the coordinate triangle is clear but what is the axis of rotation and why is it arbitrary? If arbitrary, how does an experimenter chose?

As is written in step 1.1 and the accompanying note, the arbitrary point of rotation should be chosen “such that the hypotenuse of the triangle passes through the target region.” This ultimately is how this procedure can be altered for targeting diverse brain regions.

Furthermore, we hope the inclusion of the exact formulas used to calculate the angles should be sufficient to those who have not had recent trigonometry.

How are the degrees of the angle determined?

This is at the discretion of the investigator, however, we note in the original text that “it is recommended not to exceed a coronal rotation angle of 15° due to physical constraints of the rotation apparatus.”

How is the hypotenuse calculated given only the sagittal length when the hypotenuse is necessary to determine the distance from midline "A"? How is the dorsal-ventral coordinate calculated from the hypotenuse?

Based on similar feedback from other reviewers, additional steps have been added to revised section 1 to clarify calculation of side C (hypotenuse), and suggestions for optimizing the DV coordinate.

The protocol requires rotation of the head. For many stereotaxic apparati, this is not be possible, especially given gas anesthesia. However, most apparati have the ability to rotate the needle/cannula holder relative to a fixed head. Could this be described as an alternative?

This concern has been raised by several other Reviewers. We have described this alternative in the revised Discussion and throughout the text (see above).

Minor Concerns:
The use of "sophisticated" in the title is a bit subjective, perhaps a different adjective?

The title has been altered to be less subjective, as suggested.

For 5.6 what is "reasonable"? For instance, +/- 0.02 mm?

We thank the Reviewer for pointing out this area of confusion. The text has been revised to include specific range of suitable values.

Lines 438-439, the sentence doesn't have a subject.
Line 496, hyperglycemic to hyperglycemia
Fig 5 legend, MI from the figure legend is represented as M in the fig.

The text has been corrected, accordingly.