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Free-floating Immunostaining of Mouse Brains

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TITLE:
Free-floating Immunostaining of Mouse Brains

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SUMMARY:
This protocol describes an efficient and reproducible approach for mouse brain histological studies, including perfusion, brain sectioning, free-floating immunostaining, slide mounting, and imaging.

ABSTRACT:
Immunohistochemical staining of mouse brains is a routine technique commonly used in neuroscience to investigate central mechanisms underlying the regulation of energy metabolism and other neurobiological processes. However, the quality, reliability, and reproducibility of brain histology results may vary among laboratories. For each staining experiment, it is necessary to optimize the key procedures based on differences in species, tissues, targeted proteins, and the working conditions of the reagents. This paper demonstrates a reliable workflow in detail, including intra-aortic perfusion, brain sectioning, free-floating immunostaining, tissue mounting, and imaging, which can be followed easily by researchers in this field.

Also discussed are how to modify these procedures to satisfy the individual needs of researchers. To illustrate the reliability and efficiency of this protocol, perineuronal nets were stained with biotin-labeled Wisteria florbunda agglutinin (WFA) and arginine vasopressin (AVP) with an anti-AVP antibody in the mouse brain. Finally, the critical details for the entire procedure have been addressed, and the advantages of this protocol compared to those of other protocols. Taken

together, this paper presents an optimized protocol for free-floating immunostaining of mouse brain tissue, making this process easier for beginners to follow and improve the quality, reliability, and reproducibility of these types of studies.

INTRODUCTION:

The prevalence of obesity and associated comorbidities has reached epidemic levels, causing a tremendous socioeconomic burden[1,2]. Various mouse models have been developed to better understand the biological processes responsible for obesity[3,4]. Because central mechanisms are important for the regulation of energy homeostasis in these animal models, neuroanatomical studies of mouse brains have become a necessary technique in this field. However, the quality, reliability, and reproducibility of brain histology techniques vary considerably among laboratories and even researchers within the same laboratory for various reasons (e.g., antibodies, tissues, treatments, species, research objectives). Therefore, it is necessary to establish a general protocol for histological studies of the mouse brain, including perfusion, brain sectioning, free-floating immunostaining, slide mounting, and imaging. Meanwhile, beginners can quickly learn, master, and adjust this protocol to satisfy their individual needs.

Immunohistochemical staining is an established method that has been used extensively to visualize specific cell types, mRNAs, and proteins in a variety of tissues (e.g., brain and peripheral tissues)[5,6]. More specifically, an antigen of interest can be labeled by a specific primary antibody and a corresponding secondary antibody linked to an enzyme (e.g., chromogenic immunohistochemistry) or a fluorescent dye (fluorescein isothiocyanate)[6]. As an example of the utility of these techniques, β-endorphin [one peptide encoded by pro-opiomelanocortin (POMC)] and c-fos (a marker of neuronal activity) were stained in the arcuate nucleus. Deletion of tryptophan hydroxylase 2 (an enzyme integral to serotonin synthesis) in the dorsal raphe nucleus was shown to decrease c-fos expression in POMC neurons in the arcuate nucleus[7]. In addition, the distribution of vitamin D receptor mRNA was mapped in the mouse brain via *in situ* hybridization (RNAscope)[8]. This paper presents a reliable and efficient method with a step-by-step workflow for free-floating immunostaining, aiming to improve the quality and reproducibility of histological studies of the mouse brain.

PROTOCOL:

C57BL/6J mice of both sexes (8–16 weeks of age) were used in the present study. Care of all animals and all procedures were approved by Baylor College of Medicine's Institutional Animal Care and Use Committees.

1. Perfusion

NOTE: Steps **1.1–1.6** are performed in a fume hood.

1.1. Anesthesia

1.1.1. Pour 5 mL of isoflurane (see the **Table of Materials**) onto a paper towel placed at the

bottom of a desiccator. Introduce the mouse into the desiccator and wait until signs of respiration have disappeared. Before proceeding, confirm there is no reflex to a toe-pinch.

1.1.2. Fix the mouse to a foam board by driving a pin through each foot. Ensure that the foam board is placed in a tray to collect liquid spillover.

1.2. Exposing the heart

1.2.1. Make a longitudinal superficial incision along the midline over the thorax and abdomen, then move the skin aside to expose the muscle wall of the thorax and abdomen. Next, make an incision in the muscle layer to expose the liver and the intestine. Finally, cut the rib cage with scissors to open the thorax and expose the heart and lungs. Use hemostatic forceps to pull the ribcage aside to widen the work area.

1.3. Collection of terminal blood (optional)

1.3.1. Insert a 1 mL syringe (see the **Table of Materials**) carefully into the right atrium of the heart until the tip is completely embedded. Hold the syringe steady and draw blood slowly until the desired volume is reached.

NOTE: Take care not to penetrate past the right atrium; 300–400 µL of blood is achievable per adult mouse. Additives such as a clot accelerator or anticoagulant may be used, depending on the purpose of blood collection.

1.4. Placement of the perfusion cannula

1.4.1. For beginners, cut a small hole (<1 mm) in the left ventricle of the heart with scissors and insert a blunt cannula (18 G needle for young mice and 21 G needle for aged mice) through the left ventricle into the ascending aorta. Alternatively, for experienced experimenters, penetrate the left heart ventricle with a blunt cannula directly and insert it into the ascending aorta carefully.

1.4.2. Place pins around the conjunction of the cannula and coupled tubing on the foam board to prevent movement during the perfusion. Alternatively, use hemostatic forceps to fix the cannula in place. Proceed to cut the right atrium to allow the outflow of blood from circulation.

NOTE: The perfusion cannula and coupled tubing are connected to the perfusion pump.

1.5. Perfusion with saline

1.5.1. Turn on the saline pressure switch, and perfuse the mouse transcardially with 40–60 mL of saline (0.9% NaCl: 25 °C, pH 7.4). Observe the outflow from the right atrium and the color of the liver closely.

NOTE: An automated perfusion pump (see the **Table of Materials**) is used to flush the blood within 1–2 min. As the pressure range of the pump is 1–300 mmHg, the speed can be adjusted accordingly. If saline is leaking from the nose and/or the lung is inflated, decrease the pressure and adjust the cannula. Once the outflow no longer contains blood, the brain is sufficiently flushed with saline. Meanwhile, the liver is devoid of blood and becomes brownish-gray in color.

1.6. Perfusion with formalin

1.6.1. Turn off the saline pressure switch and turn on the formalin pressure switch to perfuse the mouse with 40 mL of 10% neutral buffered formalin (10% NBF: 25 °C, pH 6.8–7.2, see the **Table of Materials**). Observe the animal's limbs for evidence of tremors.

NOTE: Tail movement may also be observed after infusion of 10% NBF. CAUTION: As NBF is biohazardous, it is suggested that researchers wear personal protective equipment (e.g., N95 mask, face shield) throughout the procedure.

1.7. Brain isolation[9]

1.7.1. Use scissors to remove the head. Make a middle line incision along the integument to expose the skull. Trim off the skin and muscle attachment with scissors.

1.7.2. Make a cut at the orbital ridge; then, place the sharp end of iris scissors into the foramen magnum. Advance the scissors along the inner surface of the skull, maintaining upward pressure to avoid damage to the brain. Remove the parietal/frontal bones and meninges carefully. Finally, remove the brain from the opened skull gently.

1.8. Post-fixation

1.8.1. Place the brain in a 15 mL tube filled with 10 mL of 5% NBF and 15% sucrose for overnight fixation at 4 °C.

NOTE: To prepare 10 mL of 5% NBF and 15% sucrose, combine 5 mL of 10% NBF and 5 mL of 30% sucrose (w/v, dissolved into phosphate-buffered saline (PBS), see the **Table of Materials**). Make sure the volume of the fixation buffer is at least 10x larger than the sample itself. Initially, the brain will float in the buffer and will sink to the bottom after overnight fixation.

1.9. Dehydration

1.9.1. Transfer the brain sample to a 15 mL tube filled with 10 mL of 30% sucrose for dehydration at 4 °C until it sinks.

2. Cryosectioning (coronal sections)

2.1. Preparation

2.1.1. Place dry ice on top of the height adjustment plate of a sliding microtome, and wait until white frost is visible. Carefully spread 5 mL of 30% sucrose on top of the plate to form a layer of a solid base after the sucrose solution is fully frozen. Place all brain samples (up to 5 brain samples in one batch) horizontally in a line on top of the sucrose base with 0.5 mL of 30% sucrose.

NOTE: The brain will immediately stick to the frozen sucrose base and gradually freeze from bottom to top. Place additional sucrose around the mounted portion of the brain to form a sturdier base for cutting.

2.2. Sectioning

2.2.1. After 5–10 min of freezing, when the brain has become hard and white, trim the brain until the desired layer/region is reached.

NOTE: Adjust the amount of dry ice on the plate to control the temperature. The temperature is too low when ice crystals appear on the surface of the brain. The temperature is too high when the brain becomes soft and is no longer white.

2.2.2 Switch from the **Trim** mode to the **Feed** mode and section brain tissue to 25 µm thickness. Prepare a 48-well plate filled with 1x PBS, and mark five wells for one mouse brain. Use a paintbrush to collect each section from one mouse and place it into one well. Collect the subsequent section of the same mouse and place it into the 2nd well.

2.2.3. Repeat 2.2.2 until the 5th well is reached, placing sections 6–10 in the 1st–5th well and so on. Repeat the same procedure for the rest of the brains simultaneously. Repeat until all the sections from one mouse are collected into the 5 wells in anatomical order.

NOTE: Following this procedure, the brain sections from each mouse are aliquoted into 5 series, which can be used for up to 5 different histological studies.

2.3. Storage

2.3.1. Use a paintbrush to transfer the sections from each well to a 1.5 mL microtube filled with cryoprotectant buffer (20% glycerol, 30% ethylene glycol, and 50% PBS), and store the samples at -20 °C.

NOTE: These sections stored in small tubes (1.5 mL) can save physical space in the freezer, and the integrity of the sections can be preserved for several months.

3. Free-floating WFA staining and anti-AVP immunostaining

3.1. Place a cell strainer into a well of a 6-well cell culture plate filled with PBS, and use a paintbrush to transfer one series of brain sections into the cell strainer. Rinse the sections in PBS

by transferring the cell strainer to another well filled with PBS. Rinse for 6 x 10 min on a shaker for sections stored in cryoprotectant buffer and 3 x 10 min for freshly cut sections.

NOTE: All the washing procedures are performed in a 6-well cell culture plate with a cell strainer inside each well (see the **Table of Materials**). Each strainer holds the brain sections of interest from each mouse. To save reagents, all the incubation procedures described below are performed in a 1.5 mL microcentrifuge tube. Between washing and incubation procedures, use a paintbrush to transfer the brain sections between each cell strainer to each tube. For washing with PBS, 12 mL is adequate for each well.

3.2. Prepare 1 mL of biotin-labeled WFA (1:1,000) solution in PBT (2.5 mL of Triton X-100 dissolved in 1,000 mL of PBS) buffer in 1.5 mL tube. Transfer brain sections from the cell strainer to the tube and incubate at room temperature on a rocking platform ~50 rpm overnight.

3.3. Rinse the brain sections with PBS for 3 x 10 min as described in 3.1.

3.4. Prepare 1 mL of streptavidin-daylight 488 (1:500) solution in PBT in a 1.5 mL tube. Transfer brain sections into the tube and incubate at room temperature for 2 h on a rocking platform at ~50 rpm.

NOTE: Cover the plate with foil to avoid light exposure from this step forward.

3.5. Rinse the brain sections with PBS for 3 x 10 min. Incubate the brain sections in blocking buffer (3% normal donkey serum diluted in PBT) for 2 h at room temperature.

NOTE: The choice of blocking serum is determined by the species from which the secondary antibody is generated. e.g., if the secondary antibody is from goat, normal goat serum should be used.

3.7. Incubate the brain sections in primary antibody (rabbit anti-AVP, 1:500) at room temperature on a rocking platform at ~50 rpm overnight.

NOTE: Prepare both primary and secondary antibodies in blocking buffer. The concentration, duration of incubation, and temperature of incubation need to be optimized in pilot studies.

3.8. Rinse the brain sections with PBS for 3 x 10 min. Incubate the brain sections in secondary antibody (Alexa Flour 594 donkey anti-rabbit IgG (H+L), 1:500) at room temperature for 2 h on a rocking platform at ~50 rpm. Rinse the brain sections with PBS for 3 x 10 min.

4. Mounting

4.1. Fill two Petri dishes (a diameter of 150 mm) with 100 mL of 1x PBS each. Transfer all the brain sections from one strainer into the first dish, and align the brain sections in neuroanatomical order from caudal to rostral.

NOTE: To avoid mixing sections from different animals, mount one series of brain sections from one animal at a time.

4.2. After all the brain sections are aligned in the first dish, submerge one slide into the second dish with one end slightly tilted with a stand (see **Figure 1** and **Figure 2**). Use a fine paintbrush to gently place a brain section just below the air-buffer interface onto the tilted slide. Repeat this process until all the desired sections are mounted onto the slide.

4.4. Use a transfer pipette to slowly and gently remove the buffer to lower its level until both brain sections are entirely above the air-buffer interface. Repeat this process until all the desired sections are mounted onto the slide.

NOTE: Take care to minimize the disturbance of the buffer surface, or the brain sections may float away. When dry, this row of brain sections will adhere to the slide firmly. If necessary, gently adjust the sections on the slide to ensure there are no wrinkles or folds in the tissue when the sections are still wet.

4.5. Repeat this process until the bottom of the slide is reached. Continue to repeat until all sections are mounted onto the slide(s).

5. Coverslipping

5.1. After all sections have dried (1–24 h at room temperature), place 80–100 µL of anti-fading mounting medium with DAPI (see the **Table of Materials**) on the slide, and gently apply a glass coverslip to cover the samples.

NOTE: Apply the glass coverslip carefully and slowly to avoid bubbles.

5.2. Place the slides in a microscope slide box (see the **Table of Materials**) and store them at 4 °C.

NOTE: Image all sections within 1–3 days to avoid fading of fluorescence and the development of autofluorescence.

6. Imaging

6.1. Turn on the scanner and the computer (see **Table of Materials**). Position the slides in the slide holder with the loading device and insert the holder in the scanner.

NOTE: The scanner enables scanning of multiple channels (e.g., 4',6-diamidino-2-phenylindole (DAPI), green, and red channels) simultaneously. The scanner scans slides only with a 20x magnification, and the Leica microscope (see **Table of Materials**) was used when a higher magnification (e.g., 40x) is needed.

6.2. Open the software for the scanner (see the **Table of Materials**). Choose the appropriate storage location and scanning profile.

6.3. Start the preview scan by clicking on the **Start Preview Scan** button. After the preview scan, open the **tissue detection** wizard and circle the regions of interest for imaging.

6.4. Click on the **Start Scan** button after choosing the regions for imaging. Wait for the machine to finish scanning. Check the result file and export the images.

NOTE: If the profile is unsuitable for the slide, adjust by refocusing and changing the exposure time or light strength to adapt the profile to the tissues. Refer to the instructions for the scanner for more technical details (**Table of Materials**).

REPRESENTATIVE RESULTS:

The flow chart of this protocol is briefly illustrated in **Figure 1**. This laboratory's cryosectioning procedure is demonstrated in **Figure 2A**, in which 5 brain samples were sectioned simultaneously. The mounting of brain sections is shown in **Figure 2B**, and a fully mounted slide with brain sections is illustrated in **Figure 2C.** In **Figure 3**, representative fluorescence immunohistochemistry images of a mouse brain section with co-staining of WFA and AVP at lower and higher magnification at Bregma -0.82 mm. AVP signals were observed in the paraventricular nucleus and the supraoptic nucleus. WFA signals were observed in the perifornical area of the anterior hypothalamus and the reticular nucleus.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow chart of fluorescence immunohistochemistry with mouse brains. Following complete anesthesia, a mouse is perfused with saline and then 10% NBF. The brain is carefully removed and cut into sections after fixation and dehydration. The sections were incubated with WFA followed by Streptavidin-daylight 488 after three washes with PBS. The brain sections were blocked and then incubated with a primary anti-AVP antibody. Then, the sections were washed 3 times with PBS followed by incubation with the secondary antibody, Alexa Flour 594 donkey anti-rabbit IgG (H+L). The brain sections were mounted on slides and coverslips placed on the slides with antifading mounting medium with DAPI before imaging. Abbreviations: NBF = neutral buffered formalin; WFA = Wisteria florbunda agglutinin; PBS = phosphate-buffered saline; AVP = arginine vasopressin; DAPI = 4',6-diamidino-2-phenylindole.

Figure 2: Photos illustrating practical cryosectioning and mounting in the laboratory. (**A**) Build up a base on top of the plate with 30% sucrose to hold all samples horizontally, cut the tissues into sections (25 µm/section) and collect the sections into a 48-well cell culture plate filled with phosphate-buffered saline. (**B**) Submerge a slide in the dish with one end slightly tilted with a stand. Place each row of sections under the air-buffer interface and lower the buffer to bring the sections out of the buffer until the bottom of the slide. The white line indicates the interface of buffer and air. (**C**) A fully mounted slide with brain sections.

Figure 3: An example of double immunofluorescent staining. (**A–D**) Microscopic images showing the distribution of WFA (red, **A**), AVP (green, **B**), DAPI (blue, **C**), and merged (**D**) in coronal mouse brain sections at Bregma -0.82 mm. Scale bars = 200 µm. (**E–H)** Higher magnification microscopic images of the white boxes in **A–D**, respectively. Scale bars = 100 µm. Abbreviations: 3V = third ventricle; PeFAH = perifornical area of the anterior hypothalamus; PVH = paraventricular nucleus; RT = reticular nucleus; SON = supraoptic nucleus.

DISCUSSION:

This protocol provides an established method for neuroanatomical studies of the mouse brain, including perfusion, tissue sectioning, free-floating immunostaining, slide mounting, and imaging. However, a few key details essential for consistent and reliable results must be optimized.

The quality of perfusion is critical for successful staining. Staining results might be affected if blood remains in the brain, given that blood cells (e.g., red blood cells) can generate an artificial 'positive' staining[10]. We infer the presence of a brownish-gray liver to indicate a high quality of perfusion, which usually results in blood-free brains. The automated perfusion pump used in this protocol helps to perfuse one animal successfully within a short period. Insufficient fixation will generate soft and fragile brain sections in the subsequent procedures, while over fixation will reduce the sensitivity of antigen reactions due to the enhanced formaldehyde cross-linking of proteins. Different conditions were tested, and overnight fixation at 4 °C was sufficient for post-fixation of mouse brains. In addition to 10% NBF used in the present protocol as a fixative buffer, 4% (w/v) of freshly prepared paraformaldehyde (PFA) in PBS has also been extensively used for tissue fixation[11].

Regarding cryosectioning, the thickness of sections needs to be decided depending on the specific needs. For instance, RNAscope studies require a thickness of 14 µm instead of 25 µm, commonly used in free-floating staining. Meanwhile, RNAscope studies require that all procedures are performed in RNAase-free solutions to preserve the integrity of the target mRNAs. Some researchers also use a section thickness of 30–40 µm for a variety of staining procedures. Conventional cryosectioning (i.e., optimal cutting temperature (O.C.T.) compound-embedded samples) allows for much thinner (e.g., 10 µm) brain sections that might be crucial for intracellular structures or other applications. The current cryosectioning strategy does not necessarily involve O.C.T. compound-embedding of brain samples and allows for 15–40 µm sections. There may be no significant difference for 3,3-diaminobenzidine (DAB) staining using 25 or 40 µm thick brain sections. However, thinner sections offer better-quality fluorescence images.

The benefit of this strategy is that multiple brain samples (up to 5 brains) can be cut at one time. However, the limitation of this method is that brain samples need to be cut within 1 week after dehydration because submersion in 30% sucrose for too long is more likely to cause protein degradation and other issues. To avoid this potential issue, these brain sections can be transferred into the cryoprotectant buffer and stored at -20 °C. For free-floating staining, the duration of incubation and concentration of both primary and secondary antibodies should be

optimized in pilot studies. Generally, overnight incubation at 4 °C or room temperature with mild shaking is appropriate for most primary antibodies, if not instructed otherwise by manufacturers. For secondary antibodies, incubation at room temperature for 1–3 h works well in most situations. However, these details must be optimized for various circumstances. For example, for c-fos staining, we typically incubate the brain sections with a concentration of 1:1,000 overnight at 4 °C for immunofluorescent staining. However, using the same antibody for c-fos DAB staining, we prefer to incubate brain sections with a concentration of 1:5,000 for 48 h at 4 °C.

A cocktail of primary antibodies and secondary antibodies might be used for double-staining to speed up the procedure. More specifically, two different primary antibodies from different species (e.g., one is from rabbit, the other one is from guinea pig, chicken, or mouse) are mixed before incubation, as are the corresponding secondary antibodies. The choice of secondary antibody is dependent on the primary antibody. If the primary antibody is from rabbit, the secondary antibody must be anti-rabbit, for example, donkey anti-rabbit or goat anti-rabbit. The selection of blocking serum depends on the secondary antibody; for example, normal donkey serum will be used if the secondary antibody is from donkey. Antigen retrieval is suggested if the immunostaining still does not work even if all guidelines have been followed strictly.

Mounting and coverslipping of brain sections must be performed in a very delicate manner. The whole process requires no wrinkles, folds, or air bubbles. It will take several trials to determine the optimal exposure time for imaging. We recommend the same exposure time for the same antibody across different sections, which is essential for comparing the signal intensities among different animals or groups. It is reasonable that exposure time might not be the same for different antibodies, even in the same section. For example, the exposure time for DAPI might be shorter than the c-fos signal in most cases.

A few procedures presented in the protocol are helpful to improve both reliability and efficiency throughout the whole process. 1) Using an automated perfusion pump for perfusion can considerably shorten perfusion time and significantly improve tissue quality. 2) This cryosectioning strategy enables slicing multiple brain samples simultaneously, which is much more efficient than conventional practice. This method is also easy for new researchers to learn and master. 3) For free-floating staining, as brain samples are stained in suspension, antibodies can penetrate the sections from both sides. We optimized the incubation strategy by placing all sections from one sample/series into a 1.5 mL microcentrifuge tube for primary and secondary antibodies, which saves antibodies, particularly when we need to stain brain samples in bulk. Another benefit of the free-floating approach is that it can be modified and applied to other histochemical staining methods (e.g., chromogenic IHC, hematoxylin and eosin, cresyl violet) in addition to immunofluorescent staining[12].

However, one limitation of free-floating staining is that very thin sections can be difficult to handle. An on-slide staining method might be considered if only a few sections need to be collected and stained immediately, as is frequently the case in clinical pathology. We also tested the on-slide staining method using brain sections generated from this protocol, and it worked well. To do this, mount the brain sections onto slides, wait for the sections to dry, and follow the

regular frozen section on-slide staining protocol. 4) Mounting free-floating sections on the slides can be tedious for certain researchers, especially for beginners. We use a fine paintbrush to gently coax sections onto the slide at the air-buffer interface and then use a transfer pipette to gently remove the buffer to lower the air-buffer interface as mounting advances from the top to the bottom of the slide. Although time-consuming, this strategy is friendly to beginners. Experienced experimenters can mount all the brains sections onto the slides in PBS at one time and only remove the buffer to bring the slide out after the last section is mounted. 5) Finally, we use a scanner for imaging, which is more efficient than fluorescence microscopy, especially when there are a large number of slides for imaging. The scanner enables scanning up to 12 slides in one batch with a 20x magnification. Alternatively, standard fluorescence microscopy can be employed in certain circumstances, for example, when a specific cluster of neurons in the brain must be showcased with a higher magnification (e.g., 40x or even 60x)[13,14].

In conclusion, this paper presents an established methodology for histological studies of mouse brains that has been proven to be reproducible, reliable, and efficient. The protocol will help generate optimal and consistent histological results among different researchers and laboratories and serve as a reference for beginners to learn this technique.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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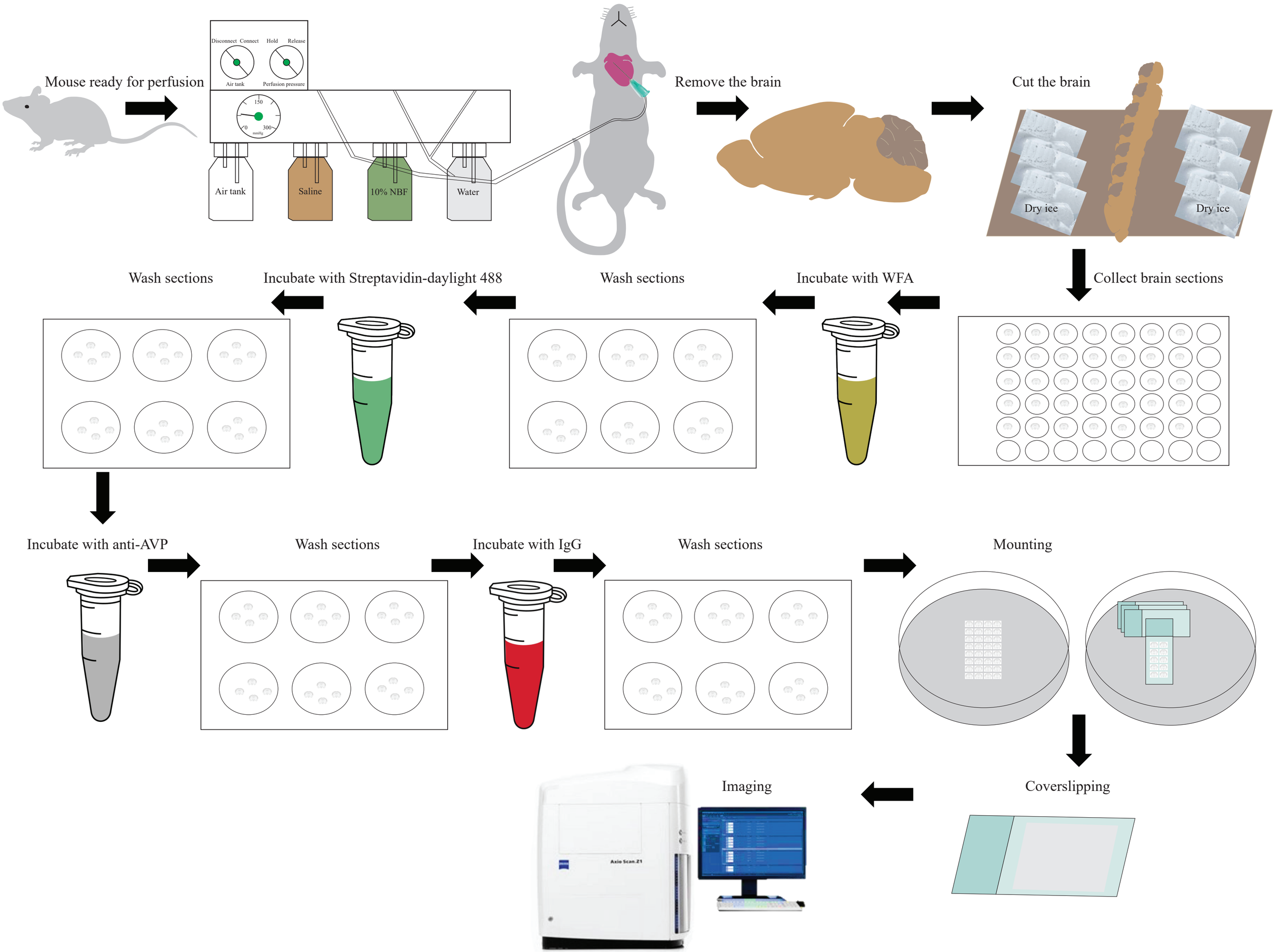

Figure 2

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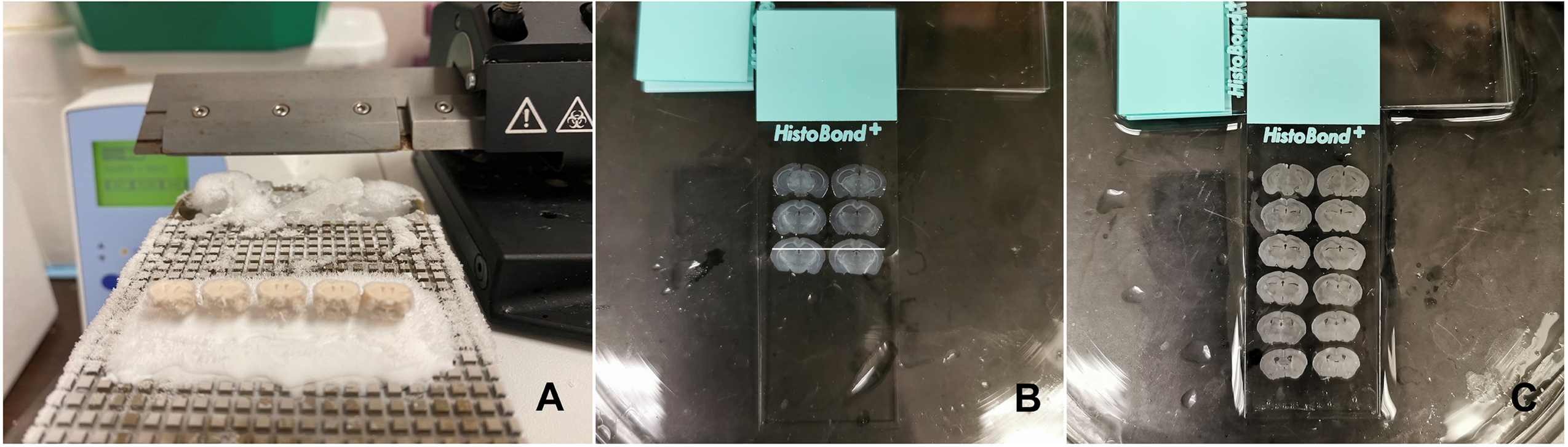
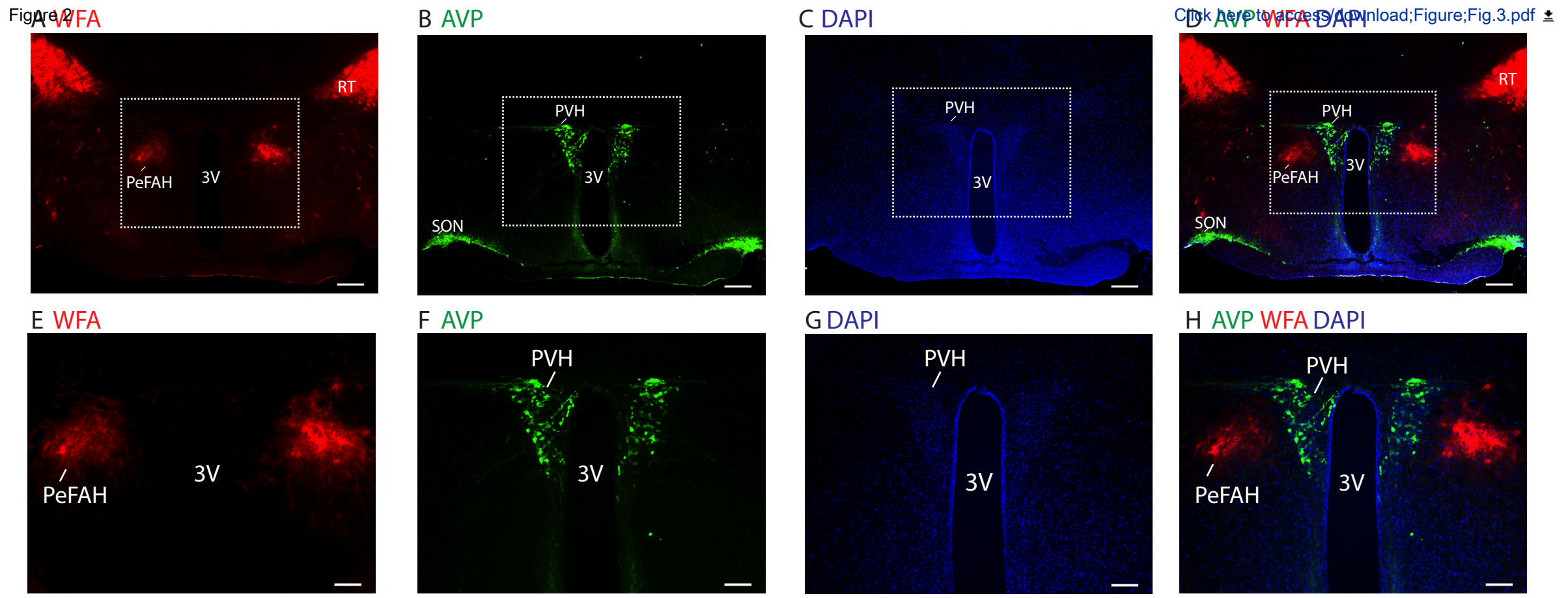


Figure 2



Dear editor,

We thank you for the expert comments/suggestions from the 3 Reviewers. All of the comments were constructive and helped us improve the quality of our submission.

A statement of all responses to all raised criticism by 3 Reviewers was provided. Moreover, the manuscript has been revised strictly according to JoVE instructions, including proofreading by two native English speakers, Dr. Jonathan Bean and Dr. Kristine Conde (who are now added as co-authors). These modifications were highlighted in **red**. We hope that our revised manuscript can now be considered suitable for publication in JoVE.

Best regards,

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Reviewers' comments:

Reviewer #4:

Manuscript Summary:

Review for Xu et al., "Routine Procedures in Neuroanatomic Analysis of Mouse Brains," describes an immunohistochemistry protocol for rodent brains. The histology protocol describes a mouse brain immunohistochemistry procedure to visualize WFA and AVA proteins.

Overall, it is a good idea to standardize immunohistochemistry experiments; however, this protocol, at this stage, is in particular useful for one the one staining described by the authors. But it can't be used as a standardized protocol since different antibodies and tissues are used in the field, and many need different treatments and procedures to make them work in the lab. It would be beneficial if the authors could provide additional information or tips and tricks to help others to optimize their protocol.

Response: We thank the reviewer for his/her comment.

We agree with the reviewer that our protocol might not be a standardized one for all immunostainings, and we changed standardized and other overstatements part in the revised manuscript accordingly. Frankly, it is extremely hard to have a standardized protocol that works for all kinds of stainings, due to different antibodies and tissues as the reviewer already mentioned, and also other reasons (e.g., species differences, different treatments, etc.). In most cases, each staining needs to be optimized before it works well. The current manuscript is meant to focus on the procedures (e.g. perfusion, sectioning, mounting, etc), rather than the optimization of antibodies and imaging analyses (which in our opinion would be extremely hard to standardize). We try our best to provide more tips and tricks in the revised manuscript such that others will be able to optimize the protocol according to their individual needs.

1) It would be helpful to provide recipes for the buffers and solutions used in this protocol where appropriate instead of ordering numbers since it is much more economical to prepare them independently, particularly for labs that have less funding.

Response: We thank the reviewer for his/her comment.

We have added the recipes for PBS, PBT, blocking serum, cryoprotectant buffer and saline preparation in the revised manuscript. For the antibodies preparation, more details are now provided in the revised manuscript. We do not prepare biohazardous substances like 10% neutral formalin ourselves in the laboratory.

2) I am a little uncertain about the key points addressed in the abstract. The authors state, for example, others ignored key details in their procedures. I agree that there is a lot of heterogeneity in the histology protocols overall in the community, which heavily impacts the reproducibility of published results. However, I do not see how the authors of this protocol address these concerns in their current manuscript. Since it describes one way to perform an antibody staining but this

can't be generalized. Further, the protocol is not yet written in a way to be flexible and adjust to other antibodies stainings. For example, it would be helpful to suggest methods for antigen retrieval, as the protocol is written now, not uniform for all different antibodies or dyes used. This histology protocol would be much more helpful for the field if it would be more flexible for different situations and not be specific for this set of antibodies since immunohistochemistry is often very individual for each experiment.

Response: We thank the reviewer for his/her comment.

We have re-written the statement that others ignored key details in the procedures that might be responsible for the heterogeneity in the revised Abstract as follows:

"However, the quality, reliability and reproducibility of brain histology results may vary among laboratories. For each individual staining, it is necessary to optimize the key procedures based on differences in species, tissues, targeted proteins, as well as the working condition of reagents" (Line 34-37)

In the revised Discussion part, we have addressed all the critical details we can think of that might affect the immunostaining results: the quality of perfusion, the duration of fixation, dehydration, thickness of brain sections, and the selection of blocking serum, primary and secondary antibody, etc. (Line317-338)

Antigen retrieval has been extensively used for paraffin-based sections, but it is very rare to do antigen retrieval for frozen brain sections that are prepared via cryosectioning. So far we have encountered only one primary antibody that did not work without antigen retrieval. We have added this suggestion in the revised manuscript (Line 361-362):

"Antigen retrieval is suggested if the immunostaining still does not work even if all guidelines have been followed strictly."

For the purpose of demonstration, we only show the double immunostaining of WFA and AVP in the manuscript. Obviously, other antibodies can also be applied based on this protocol. We have tested a large number of different primary antibodies in the laboratory for various research aims. However, working condition of reagents, particularly for primary antibody, need to be optimized for each experiment before starting the experiment.

We have removed the word flexibility regarding our protocol in the revised manuscript as it might not be that flexible for other histochemical stainings such as chromogenic IHC, hematoxylin and eosin, cresyl violet, given that our protocol is to serve a good example for beginners to learn this technique.

3) This protocol could provide much more guidance. For example, you can indeed section multiple brains using a microtome, but cryosectioning allows much thinner brain sections that might be crucial for intracellular structures or other applications.

Response: We agree with the reviewer that cryosectioning allows much thinner sections like 10 µm or even 6 µm, and microtome allows >15µm according to our experience. For brain sections, free floating staining method we used is compatible with thicker sections, and 25-40 µm is the most acceptable and reasonable for a variety of immunostainings; while for brain sections used in RNAscope studies, we typically use 12-15 µm.

We have added this point in the Discussion: "Conventional cryosectioning (i.e., O.C.T. embedded samples) allows for much thinner (e.g. 10 µm) brain sections that might be crucial for intracellular structures or other applications." (Line 332-334)

4) Imaging section and data analyses should be more detailed.

Response: We thank the reviewer for his/her comment.

We have provided more details as to the imaging via scanner in the revised manuscript as follows:

"**6 Imaging**

6.1. Turn on the scanner and the computer (see Table of Materials). Position the slides in the slide holder with the loading device and insert the holder into the scanner.

Note: The scanner enables scanning of multiple channels (e.g., DAPI, green and red channel) simultaneously. The scanner scans slides only with a 20X magnification, and the Leica microscope (see Table of Materials) can be employed when a higher magnification (e.g., 40X) is needed.

6.2 Open the ZEN 3.1 software for the scanner. Choose the appropriate storage location and scanning profile.

6.3 Start the preview scan by clicking the "Start Preview Scan" button. After preview scan, open the tissue detection wizard and circle the regions of interest you want to image.

6.4 Click the "Start Scan" button after choosing your regions to image. Wait for the machine to finish scanning.

6.4 Check the result file and export the images."

Note: If the profile is not suitable for the slide, adjust by refocusing, changing the exposure time or light strength to adapt the profile to your tissues. Refer to the instruction of Zeiss Axio Z1 Scanner for more technical details." (Line 257-274)

We decided to remove the data analyses parts in the revised manuscript, as the overarching goal of this protocol is to demonstrate perfusion, sectioning and free-floating staining and imaging of mice brain samples. We do not intend to provide guidelines for data analyses, because we felt it would be extremely hard to standardize the data analyses which can vary based on a lot of factors,

e.g. the purposes of studies, the antibodies (the combinations of antibodies) used, the regions of interest in the brain, etc.

Reviewer #5:

Manuscript Summary:

This protocol paper entitled 'Routine Proceduers in Histological Studies of Mouse brains' introduced and described a step-by-step method for histological analysis in mouse brains, especially free-floating immunostaining. The authors improved the manuscript significantly according to the reviewers' comments and suggestions, but there are still several major and minor points to be addressed.

Major Concerns:

Title: this paper does not cover broad ranges of the procedures in histological analysis. Instead, it is highly focused on the free-floating staining methods. Therefore, I strongly recommend revising the title again to specify and emphasize the aim of this protocol so that readers can easily find what they want.

Response: We thank the reviewer very much for his/her suggestion.

We have now changed the title into “Routine Procedures in Free-floating Immunostaining of Mouse Brains”, which is more specific to the aim of this protocol.

Protocol

Step 1.3: please add storage condition after taking out blood. For example, mix the blood with EDTA to prevent agglutination.

Response: We thank the reviewer for his/her comment.

We collect terminal blood to measure hormonal concentrations such as leptin, insulin, prolactin, etc. In this case, we do not mix blood with EDTA as we only need serum samples separated from the whole blood.

We now extend Step 1.3 in the revised manuscript as follows:

“1.3 Collection of terminal blood (optional)

1.3.1 Insert a 1 ml syringe (See Table of Materials) carefully into the right atrium of the heart until the tip is completely embedded. Hold the syringe steady and draw the blood slowly until desired volume is reached.

Note: Take care not to penetrate past the right atrium. 300-400 µl of blood is achievable per adult mouse. Additives such as a clot accelerator or anticoagulant may be used, depending on the purpose of blood collection.” (Line 97-104)

Step 1.4: In my experience, it is too risky to cut a small hole in the left ventricle with a scissor,

which can abrupt leakage of blood from the heart. This procedure can make it hard to find the incision point quickly. Please revise the method, or add an alternative method, if possible. Also, 21G needle is too big for 2-3 months of age mice. Please describe it as 18G for young mice and 21 G for aged mice.

Response: We thank the reviewer for his/her comment.

We have revised Step 1.4 in the revised manuscript as follows:

"1.4 Placement of the perfusion cannula.

1.4.1. For beginners, cut a small hole (< 1 mm) in the left ventricle of the heart with scissors and insert a blunt cannula (18 G needle for young mice and 21 G needle for aged mice) through the left ventricle into the ascending aorta. Alternatively, for experienced experimenters, penetrate the left heart ventricle with a blunt cannula directly and insert it into the ascending aorta carefully.

1.4.2. Place pins around the conjunction of the cannula and coupled tubing on the foam board to prevent movement during the perfusion. Alternatively, use hemostatic forceps to fix the cannula in place. Proceed to cut the right atrium to allow the outflow of blood from circulation.

Note: The perfusion cannula and coupled tubing are connected to the perfusion pump." (Line 105-115)

Step 1.5: Please specify the composition of the perfusion solution. If the mouse was perfused with saline followed by a fixative, both solutions should contain anticoagulants to prevent blood clots. Please revise it.

Response: We thank the reviewer for his/her comment.

We only collect terminal blood before perfusion, see Step 1.3. Therefore, there is no need to contain anticoagulants in the saline or 10% NBF during perfusion. Blood clot in blood vessels is not a problem for perfusion with the pressure pump as blood will be flushed out from the circulation within a short period of time. We are able to finish perfusion of one mouse within 3-4 minutes (1-2 min for saline, 1-2 min for 10% NBF).

Step 1.6: please add that this procedure should be performed under the fume hood.

Response: We thank the reviewer for his/her comment.

Step 1.1 to 1.6 are performed in the fume hood as outlined already in the manuscript (see Line 84)

Step 2.2: it would be very helpful if the authors can add more details about the practical method

for putting or storing multiple brains in the tube or 48 well-plate during the following procedures. It is hard for me to visualize this step clearly.

Response: We thank the reviewer for his/her comment.

We do our utmost to use simple words to describe the procedure. However, it is reasonable that readers, particularly researchers who do not have much direct experience, might not clearly follow all the details. We believe it will make more sense when the writing is accompanied with the video that we will prepare with help of the JoVE staff.

We now modified Step 2.2 in the revised manuscript as follows:

"2.2 Sectioning.

2.2.1. After 5 to 10 min of freezing, the brain will become hard and white. When the brain is completely frozen, trim the brain until the desired layer/region is reached.

Note: Adjust the amount of dry ice on the plate to control the temperature. The temperature is too low when ice crystals appear on the surface of the brain. The temperature is too high when the brain becomes soft and is no longer white.

2.2.2 Switch from the "Trim" mode to the "Feed" mode and section brain tissue into (25 µm/section). Prepare a 48-well plate filled with 1x PBS solution, and mark five wells for one mouse brain. Use a paintbrush to collect each section from one mouse and place it into one well. Collect the subsequent section of the same mouse and place it into the 2nd well. Repeat this procedure until the 5th well is reached. Sections 6-10 will be put into the 1st-5th well and so on. Repeat the same procedure for the rest of brains simultaneously. Repeat until all the sections from one mouse are collected into the 5 wells in an anatomical order.

Note: Following this procedure, the brain sections from each mouse are aliquoted into 5 series, which can be used for up to 5 different histological studies." (Line 163-179)

Step 3: please specify the volume of solution that is used for washing or staining.

Response: We thank the reviewer very much for spotting the omission.

The volume of solution and duration for washing or staining is now provided in the revised manuscript. (Line 191, Line 192, Line 204, Line 208, Line 220 and Line223)

Step 6: The imaging part is also a very critical step for histological analysis, especially, when multiple brains are analyzed simultaneously. The authors introduced Zeiss Axio Scan, Z1 and Leica microscope without any detailed protocol to achieve rigorous results from multiple samples. Please elaborate this part more.

Response: We thank the reviewer for his/her comment.

We decide to remove the data analyses part in the revised manuscript. We replied to the same question raised by reviewer 4#: the overarching goal of this protocol is sectioning and free-floating staining of mice brain samples. We do not intend to provide guidelines for data analyses, because we felt it would be extremely hard to standardize the data analyses which can vary based on a lot of factors, e.g. the purposes of studies, the antibodies (the combinations of antibodies) used, the regions of interest, etc.

We have provided more details as to the imaging via scanner in the revised manuscript as follows:

"Imaging

6.1. Turn on the scanner and the computer (see Table of Materials). Position the slides in the slide holder with the loading device and insert the holder into the scanner.

Note: The scanner enables scanning of multiple channels (e.g., DAPI, green and red channel) simultaneously. The scanner scans slides only with a 20X magnification, and the Leica microscope (see Table of Materials) can be employed when a higher magnification (e.g., 40X) is needed.

6.2 Open the ZEN 3.1 software for the scanner. Choose the appropriate storage location and scanning profile.

6.3 Start the preview scan by clicking the "Start Preview Scan" button. After preview scan, open the tissue detection wizard and circle the regions of interest you want to image.

6.4 Click the "Start Scan" button after choosing your regions to image. Wait for the machine to finish scanning.

6.4 Check the result file and export the images.

Note: If the profile is not suitable for the slide, adjust by refocusing, changing the exposure time or light strength to adapt the profile to your tissues. Refer to the instruction of Zeiss Axio Z1 Scanner for more technical details." (Line 257-274)

Discussion:
The authors said, 'this protocol provides a comprehensive procedure for neuroanatomical studies of…'. However, there is no specific procedure for neuroanatomical analysis in this protocol. Please provide an actual protocol or procedure regarding neuroanatomical analysis or describe specifically what this protocol provides.

Response: We thank the reviewer for his/her comment.

We have removed the analysis part in the protocol as our focus is perfusion, sectioning and free-floating, mounting and imaging.

This protocol uses a free-floating system. However, most labs routinely perform frozen brain cryosectioning or praffinized brain sectioning. Please add more description about the advantage and disadvantages of a free-floating system compared to other methods.

Response: We thank the reviewer for his/her comment.

We have extended this part in this discussion part.

"As to free-floating staining, brain samples are stained in suspension, and therefore antibodies can penetrate the sections from both sides. We optimized the incubation strategy by placing all sections from one sample/series into a 1.5 ml Eppendorf tube for both primary and secondary antibody, which saves antibodies, particularly when we need to stain brain samples in bulk. Another benefit of the free-floating approach is that it can be modified and applied to other histochemical staining (e.g., chromogenic IHC, hematoxylin and eosin, cresyl violet) in addition to immunofluorescent staining12. However, one limitation of free-floating staining is that very thin sections can be difficult to handle. An on-slide staining method might be considered if there are only few sections that need to be collected and stained immediately like in clinical pathology. We also tested the on-slide staining method using brain sections generated from our protocol and it worked well. To do this, mount the brain sections onto slides and wait for the sections to dry, then follow the regular frozen section on-slide staining protocol." (Line 376-388)

The authors described other types of staining in this section. In other words, the authors only provide protocol and expected outcome for WFA/AVP staining. It would be helpful if the authors can provide more examples about staining procedures and results for other types of staining.

Response: We thank the reviewer for his/her comment.

Yes, we did mention other types of staining, like DAB staining and RNAscope. While these two kinds of stainings were demonstrated in our recent publications (see ref 7 and 8). DAB staining (e.g., c-fos) is similar to WFA/AVP more or less as we demonstrated in the protocol. While RNAscope is totally different, and it deserves a separate protocol. It is hard to integrate all exact steps of DAB staining and RNAscope in this protocol as it would make it very lengthy and unclear, particularly for learners. Therefore, we would like to keep the protocol easy, clean and succinct.

Justification, economical option, for the advantages of the authors' method is very week. Please provide more scientific reasons.

Response: We thank the reviewer for his/her comment.

We have removed the statement upon economical option, and extended more scientific reasons in the revised Discussion part.

"A few procedures presented in the protocol are helpful to improve both reliability and efficiency throughout the whole process. 1) Using an automated perfusion pump for perfusion can greatly

shorten perfusion time and significantly improve tissue quality. 2) Our cryosectioning strategy enables slicing multiple brain samples at one time, which is much more efficient compared to the conventional practice. This method is also easy for new researchers to learn and master. 3) As to free-floating staining, brain samples are stained in suspension, and therefore antibodies can penetrate the sections from both sides. We optimized the incubation strategy by placing all sections from one sample/series into a 1.5 ml Eppendorf tube for both primary and secondary antibody, which saves antibodies, particularly when we need to stain brain samples in bulk. Another benefit of the free-floating approach is that it can be modified and applied to other histochemical staining (e.g., chromogenic IHC, hematoxylin and eosin, cresyl violet) in addition to immunofluorescent staining12. However, one limitation of free-floating staining is that very thin sections can be difficult to handle. An on-slide staining method might be considered if there are only few sections that need to be collected and stained immediately like in clinical pathology. We also tested the on-slide staining method using brain sections generated from our protocol and it worked well. To do this, mount the brain sections onto slides and wait for the sections to dry, then follow the regular frozen section on-slide staining protocol. 4) Mounting free-floating sections on the slides can be tedious for certain researchers, especially for beginners. We use a fine paintbrush to gently coax sections to the slide at the air-buffer interphase, and then use a transfer pipette to gently remove the buffer to lower the air-buffer interface as mounting advances from the top to the bottom of the slide. Although time consuming, this strategy is friendly to beginners. Experienced experimenters can mount all the brains sections to the slides in PBS at one time, and only remove the buffer to bring the slide out after the last section is mounted. 5) Finally, we use a scanner for imaging, which is more efficient than fluorescence microscopy, especially when we have a large number of slides for imaging. The scanner enables us to scan up to 12 slides in one batch with a 20X magnification. Alternatively, regular fluorescence microscopy can be employed in certain circumstances, for example, when we need to showcase a specific cluster of neurons in the brain with a higher magnification (e.g., 40X even 60X)13,14." (Line 372-400)

Reviewer #6:

Manuscript Summary:

This methods paper covers an important topic that is used across broad fields in Neuroscience. Strict protocols are important because studies involving antibodies are some of the most unreliable in science. While the authors were responsive to the previous reviewer comments, it is still not publishable in the current form. There writing is hard to follow at times, there are grammatical errors throughout, and the microscopy parts of this paper are weak and show a lack of expertise in this area that should noted as a limitation.

Major/Minor Concerns:

Abstract: 'details' should be changed to 'detail'.

Response: We thank the reviewer for spotting the error.

Details has been changed into detail (Line 38).

Introduction:

The first sentence two sentences are not relevant to this paper and should be deleted. This technique is a broadly used method that is not specific to obesity or research on metabolic syndromes. This is the same technique used by laboratories studying development, neurodegenerative diseases, brain injury etc. The title of this paper reflects this technique is generalizable across multiple disciplines.

Response: JoVE is producing a collection of video articles on Tools to Characterize Energy Metabolism in Animal Models. Based on our work in obesity and other metabolic syndrome, we are invited to draft up this protocol showcasing recent research methods relevant to metabolism in this field. Thus, we need to keep the first few sentences as necessary background that matches this collection.

Line 5, delete the word 'the' in front of 'neuroanatomical studies' and the word 'has' should be 'have'.

Response: We thank the reviewer for spotting the error.

We now revised it as follows:

"Since central mechanisms are important for the regulation of energy homeostasis in these animal models, neuroanatomical studies of mouse brains have become a necessary technique in this field" (Line 52-54)

'for certain reasons' is ominous and vague. This is the entire premise for the paper and should be clearly stated. What mistakes are other labs making? What is leading to variability in staining protocols?

Response: We thank the reviewer for question.

We now have extended it in the revised manuscript as follows:

"However, the quality, reliability and reproducibility of brain histology techniques vary considerably among laboratories and even researchers within the same laboratory for a variety of reasons (e.g., antibodies, tissues, treatments, species, research purposes. etc.)." (Line 55-57)

Line 8-9 there is a grammatical error with 'standardized protocol of histological studies'. Should 'of' be 'for'? "standardized protocol for histological studies…"

Response: We thank the reviewer for spotting the error.

"Of" has been replaced by "for" in the revised manuscript. (Line 58)

The sentence that starts, 'in essence' is unclear and grammatically incorrect. This is the main

focus of the entire paper and should be written clearly and highlight the importance of this work. Delete, "Based on the experience we have earned…". It is grammatical incorrect, and it highlights that the authors only have two publications with this technique (there are labs with 100s of papers cutting and staining tissue).

Response: We thank the reviewer for spotting the error.

We have modified the second paragraph of Introduction as follows:

"Immunohistochemistry staining is an established method that has been used extensively to visualize specific cell types, mRNAs and proteins in a variety of tissues (e.g., brain and peripheral tissues)[5,6]. More specifically, an antigen of interest can be labeled and revealed by a specific primary antibody and corresponding secondary antibody that are either linked to an enzyme (e.g., chromogenic immunohistochemistry) or a fluorescent dye (fluorescein isothiocyanate)6. As an example of the utility of these techniques, we have previously stained β-endorphin [one peptide encoded by pro-opiomelanocortin (POMC)] and c-fos (a marker of neuronal activity) in the arcuate nucleus. We were then able to demonstrate that deletion of tryptophan hydroxylase 2 (an enzyme integral to serotonin synthesis) in the dorsal raphe nucleus can decrease c-fos expression in POMC neurons in the arcuate nucleus[7]. In addition, we mapped the distribution of vitamin D receptor mRNA in the mouse brain via in situ hybridization (RNAscope)[8]. Here we present a reliable and efficient method with a step-by-step workflow for free-floating immunostaining, aiming to improve the quality and reproducibility of histological studies of the mouse brain." (Line 63-76)

The introduction states this is for mouse brains. Wouldn't the same technique apply to rats?

Response: We thank the reviewer for the question.

Though we do not have extensive experience with rat brains, it is conceivable that our procedures can be applied to rat brains, of course with some modifications in certain steps. For example, the multiple brain sectioning may not be done with as many brains all together and the free-floating incubations may need to be done in bigger tubes than 1.5 ml, given the larger sizes of rat brains.

Protocol:
Delete the word 'present'. The sentence should just say, 'used in this study'.

Response: we do not agree with the reviewer. There is no doubt that *present* can be used here from the perspective of grammar.

Section 1.1 Anesthesia: do you actually put 5mL of isoflurane in the bottom of the box? This is unclear. If it is a solution, then what is the solution? What is the iso in/on? Iso can burn the skin of a mouse, is it on gauze? More details are needed for this section. Checking the mouse every minute is not often enough. Mice should be checked every 10 seconds using this method of

anesthesia which has a rapid onset of effects. More detail about how the mouse should be fixed to the board. Details are for people who have never done this method, where are you placing these pins? Through the hands and feet? What is 'surgical phase of anesthesia'? This is not clear and not a commonly used term. The word 'operated' should be 'performed'.

Response: We thank the reviewer for the question.

Isoflurane is a non-explosive, non-flammable, inhaled general antithetic, and of course it is a solution. That is to say isoflurane itself is solution – each ml contains 99.9% isoflurane. As we mentioned in the manuscript, we generally fill 5 ml isoflurane solution *at the bottom of* the autoclavable plastic desiccator, in which there is aluminum plate that is used to hold animals, and separate animals from contacting liquid isoflurane. We do not check the mouse every 10 seconds, because this is terminal experiment. We will wait till the mouse losses both pain sensation and breath, but still keeps heart beating. Generally it takes 1-3 mins on average due to individual variation.

We have amended Step 1.1 in the revised manuscript as follows:

"1.1 Anesthesia

1.1.1 Pour 5 ml of isoflurane (See Table of Materials) onto a paper towel placed at the bottom of a desiccator. Introduce the mouse to the desiccator and wait until signs of respiration have disappeared. Before proceeding, confirm there is no reflex to a toe-pinch. The mouse can now be fixed to a foam board by driving a pin through each foot. The foam board should be placed in a tray to collect liquid spillover." (Line 85-90)

Section 1.4: 'with a scissor' should be changed to 'with scissors'. 'use a hemostatic forceps' should be 'use hemostatic forceps'. 'the flow-out of blood from circulation' is not clear. 'to allow blood from circulation to flow out'.

Response: We thank the review for spotting the error.

We have re-written Section 1.4 as follows:

"1.4 Placement of the perfusion cannula.

1.4.1. For beginners, cut a small hole (< 1 mm) in the left ventricle of the heart with scissors and insert a blunt cannula (18 G needle for young mice and 21 G needle for aged mice) through the left ventricle into the ascending aorta. Alternatively, for experienced experimenters, penetrate the left heart ventricle with a blunt cannula directly and insert it into the ascending aorta carefully.

1.4.2. Place pins around the conjunction of the cannula and coupled tubing on the foam board to prevent movement during the perfusion. Alternatively, use hemostatic forceps to fix the cannula in place. Proceed to cut the right atrium to allow the outflow of blood from circulation.

Note: The perfusion cannula and coupled tubing are connected to the perfusion pump." (Line 105-115)

Section 1.5: This is very hard to follow. The first sentence should be re-written, it is not clear. While the liver clears while blood is replaced with saline, so does every other organ. This is misleading. As a note you can add that liver is a good visual cue that be used. Is Automated Perfusion Pump the brand? If not, there is no need to capitalize. "Instead of gravity" is not clear. Gravity could not be used to pump saline around the mouse body. Be clear you are referring to a manual gravity perfusion, which is a technique, not the actual use of just gravity. More details should be added about the speed that is used. This is a step that can introduce variability in tissue quality and rupture vessels. The authors state, 'the speed can be adjusted accordingly' but how fast is optimum, on what parameters do you make this decision? Does the size of the mouse or blood volume need to be considered?

Response: We thank the reviewer for the questions.

We have now re-written Step 1.5 in the revised manuscript:

"1.5 Perfusion with saline

1.5.1. Turn on saline pressure switch, and perfuse the mouse transcardially with 40-60 ml saline (0.9% NaCl: 25 °C, pH 7.4). Observe the outflow from the right atrium and the color of the liver closely.

Note: An automated perfusion pump (see Table of Materials) is used to flush the blood within 1-2 min. The pressure range of the pump is 1 to 300 mmHg, therefore the speed can be adjusted accordingly. If saline is leaking from the nose and/or the lung is inflated, decrease the pressure and adjust the cannula. Once the outflow no longer contains blood, the brain is sufficiently flushed with saline. Meanwhile, the liver is devoid of blood and becomes brownish-gray in color. " (Line 116-125)

As we already mentioned in the original manuscript, the pressure range induced by this pump is between 1 to 300 mmHg, and our practice is pressure range150 to 300 mmHg works fast and well for both saline and 10% NBF. However, it does not matter that much if one prefers 100 mmHg throughout the whole process.

Obviously, gravity has also been used to pump saline and formalin for perfusion. Hang the bottle of saline at a higher point than mouse (the higher, the better), then gravity induces saline around mouse. The same for formalin. The disadvantage of this method is the pressure induced by the difference of height between bottle and mouse sometime is not enough.

For the automated perfusion pump, the speed is not that strict, it should take a bit longer time with low pressure like 100 mmHg, but shorter time with a higher pressure such as 300 mmHg.

The size of mouse or the volume of blood might be considered. However, mice in our laboratory (20-60 g) have been perfused very well so far.

Section 1.6: First sentence needs to be re-written for clarity and grammar. Can paraformaldehyde be used in the place of formalin? There are inconsistencies throughout with formalin/para. Tail suspension? Be more detailed. Do you mean movements in the tail? 'indicating that fixation is ongoing through the whole circulation' should be re-written for clarity and grammar.

Response: We thank the reviewer for the question.

We did not mention paraformaldehyde here, although 4% PFA solution has also been widely used for perfusion and fixation for brain and other tissues in a variety of species. (Line 325-328)

We have modified Step 1.6 in the manuscript as follows:

"1.6 Perfusion with formalin.

1.6.1 Turn off the saline pressure switch and turn on the formalin pressure switch to perfuse the mouse with 40 ml of 10% neutral buffered formalin (10% NBF: 25 °C, pH 6.8-7.2, see Table of Materials). Observe the animal's limbs for evidence of tremors.

Note: Tail movement may also be observed after infusion of 10% NBF. CAUTION: Since NBF is biohazardous, it is suggested that researchers wear personal protective equipment (e.g., N95 mask, face shield) throughout the procedure." (Line 126-133)

Section 1.7: Remove the head, how? A note should be added to this step that inserting the scissors and advancing them is a very delicate step and care should be taken. How do you remove the parietal and frontal bones and meninges? What tool do you use? How do you take out the brain? What tool do you use? Do you cut the cranial nerves?

Response: We thank the reviewer for the question.

One pair of scissors (e.g., Iris scissors, 10 cm, angled) as well as curved forceps (152 mm L, curved serrate tips) is enough for brain dissection, at least for mouse. It does need other tools for large species like ferrets. We do not need to cut cranial nerves specifically.

We did not intend to elaborate brain isolation in that great detail as this has been clearly and fully demonstrated in Ref 9. A video will be more helpful than writing here, so are for other procedures in the protocol.

Section 1.8: The brain will sink post-fixation is vague. What is an approximation for how long this should take? A day or a month? It is not advised to mix your sucrose in formalin, this can over-fix your tissue.

Response: We thank the reviewer for the question.

The brain will float at the beginning and sink at the bottom after post-fixation, simply because there are half of sucrose (volume) for fixative buffer. As we already mentioned in the original manuscript, we fix the samples overnight at 4 °C (Line142-143). With this overnight post-fixation procedure, we have never encountered the over-fix issue.

Section 2.1: How are you cutting 5 brains? How are you delineating between the samples? Not sure what this sentence means, "Carefully spread 5ml 30% sucrose on top of plate, and a layer of solid base will be formed after sucrose solution is fully frozen".

Response: We thank the reviewer for the question.

We have modified Step 2.1 in the revised manuscript as follows:

"2.1. Preparation.

2.1.1 Place dry ice on top of the height adjustment plate of a sliding microtome, and wait until white frost is visible. Carefully spread 5 ml of 30% sucrose on top of the plate to form a layer of solid base after the sucrose solution is fully frozen. Place all brain samples (up to 5 brain samples in one batch) horizontally in a line on top of the sucrose base with 0.5ml of 30% sucrose.

Note: The brain will stick to the frozen sucrose base immediately and will gradually freeze from bottom to top. Place additional sucrose around the mounted portion of the brain to form a sturdier base for cutting." (Line154-162)

We do believe a video will be very helpful for understanding how exactly we do it.

Section 2.2: This section is unclear and could not be followed by a person with new to this technique. Are the brains turning white or the freezing medium? Delete the word tissues, 'Cut brain tissue into sections'. An optimal cutting temperature should be kept the entire time. Varying cutting temperature is bad for the tissue and not a good scientific practice, this should be deleted. 'Remove some dry ice if brain samples are too hard; add more dry ice if the brains become soft.' This is not an ideal practice and not informative for a scientist new to this technique. What is 'too hard'?

Response: We thank the reviewer for the question.

We would say brains are turning white as there are ice crystals appearing on the surface. Freezing medium (i.e., sucrose) is only dropped at the bottom where the brain attaches to the solid base that is made up of frozen sucrose.

It is true that optimal cutting temperature should be kept during the entire time. In reality, the temperature needs to be kept within a small range, like -20 to -25 °C, rather than an exact number like -21 °C. And this is actually how cryosectioning works. For example, when brain sample is

over frozen, it indicates the temperature is too low, or versus. Unlike cryosectioning, our sectioning strategy is not performed in a confined space, but in an open space. Therefore, we adjust the temperature by removing or adding dry ice, and this works well so far.

Section 2.3: No fridge is at -20. Sections can be stored one year but it is a BOLD statement to say the immunostaining would not be affected. If you stained fresh tissue next to one year old tissue, there would certainly be a difference in most stains.

Response: We thank the reviewer for the question.

We have modified Step 2.3 in the revised manuscript as follow:

"2.3 Storage.

Use a paintbrush to transfer the sections from each well to a 1.5 ml microtube filled with cryoprotectant buffer (20% glycerol, 30% ethylene glycol and 50% PBS), and store the samples at -20 °C.

Note: These sections stored in small tubes (1.5 ml) can save physical space in the freezer, and the integrity of the sections can be preserved for several months." (Line 180-183)

Section 3.1: Be consistent with reporting time (6x for 10 minutes vs 3 x 10 min).

Response: We thank the reviewer for spotting the error.

This has been corrected in the revised manuscript. (Line 191, Line 192, Line 204, Line 208, Line 220 and Line223)

Section 3.4: What is PBT? What is WFA? 'Prepared into a' should just be 'prepared in'. 'Avoid light exposure from this step' should be 'for this step'.

Response: We thank the reviewer for the question, and we have added the annotation of the abbreviations.

As mentioned in the original manuscript, the preparation of PBT is 2.5 ml Triton X-100 dissolved into 1000 ml PBS. (Line 200-201).

WFA is short for Wisteria florbunda agglutinin (Line 42), which has been used to specifically labeled distribution of perineuronal nets in the brain.

Light exposure needs to be avoided from Step 3.4 forward to the end of the staining due to fluorescence secondary antibody. Therefore, we need to keep avoiding light exposure from this step, not for this step only.

Section 3.6: For almost all antibodies steps like the blocking require different concentrations of reagents for them to work optimally. This protocol does not reflect the fact that steps should be optimized before starting experiments. Details on how to match the blocking species to the experiment should also be included.

Response: We thank the reviewer for raising an excellent point.

For the blocking procedure, our practice is 3% normal serum (donkey or goat normal serum) for 2-3 hours at room temperature which works well at least for mouse brain sections.

The antibodies, particularly primary antibodies, need to be optimized such as concentration and duration, incubated at room temperature or 4 °C.

The choice of blocking serum is determined by the species from which the second antibody is generated. For example, if goat anti-rabbit secondary antibody is used, use goat normal serum. If donkey anti-chicken secondary antibody is used, then use donkey normal serum accordingly.

We have modified Step 3.6 in the revised manuscript as follows:

"3.6. Incubate the brain sections in blocking buffer (3% normal donkey serum diluted in PBT) for 2 hours at room temperature.

Notes: The choice of blocking serum is determined by the species from which the secondary antibody is generated. e.g., if the secondary antibody is from goat, goat normal serum should be used." (Line 209-214)

Section 3.7 : 'and transfer all the sections of interest into the tube for incubation.' This is unclear, this is saying put all sections in the study into one tube. How are you supposed to know what animal they are from or what treatment group? This is extremely misleading. 'Prepare both primary and secondary antibody in a 1.5 mL Eppendorf tubes' should be 'tube'.

Response: We thank the reviewer for the question.

Putting all sections of interest from *one single animal*, not all animals, into one tube is actually we do in the laboratory. There are 5 series (each series/tube) for *one single animal*, and we use one series for each staining. We have made it more clearly in the revised manuscript as follows:

"3.7. Incubate the brain sections in primary antibody (rabbit anti-AVP, 1:500) at room temperature on a rocking platform ~50 rpm overnight.

Note: Prepare both primary and secondary antibody in blocking buffer. The concentration, duration of incubation and temperature of incubation need to be optimized in pilot studies." (Line 215-219)

Section 3.9: Should 'Alexa Fluo 594' should be 'Alexa Flour 595'? It is necessary to add a note that these concentrations should be modified based on the antibody/tissue/study.

Response: We thank the reviewer for spotting the error.

We have amended Step 3.9 in the revised manuscript as follows:

"3.9. Incubate the brain sections in secondary antibody (Alexa Flour 594 donkey anti-rabbit IgG (H+L), 1:500) at room temperature for 2 hours on a rocking platform ~50 rpm." (Line 221-222)

A note has been added in Step 3.7.

Section 4.1: It is unclear what is meant by, 'Prepare two Petri dishes with a diameter of 150 mm filled with100 ml1x PBS individually.' This section says line the brain sections in the neuroanatomical order from caudal to rostral, but a previous section says put them all in one well. How is someone supposed to know which order is correct by eye? How do you line them in the dish without them floating around? What is the purpose of the two petri dishes, it is not clear?

Response: We thank the reviewer for the questions.

In previous steps, we put all sections from one animal in one well for incubation and wash. When it comes to mounting, we firstly transfer all sections from each one well to a Petri dish, which is filled with 100 ml PBS, and then line these brain sections in the neuroanatomical order. To avoid mixing with other sections from other animals, we transfer and mount sections from each animal at one time. The other dish is used to mount sections on the slide after all sections have been lined well in the first dish (See Figure 1). To line all brain sections in the neuroanatomical order takes lot of practice. Likewise, lining and mounting sections on the slides quickly and reliably take time and practice.

We have amended Step 4.1 in the revised manuscript as follows:

"4.1. Fill two Petri dishes (a diameter of 150 mm) with 100 ml 1x PBS, each. Transfer all the brain sections from one strainer into the first dish, and align the brain sections in neuroanatomical order from caudal to rostral.

Note: To avoid mixing sections from different animals, mount one series of brain sections from one animal at a time." (Line 226-231)

4.2: This is unclear. No idea what this means. 4.3: Unclear. Tap a brain section below the air-buffer interface?? 4.4: This step is unclear. It says suck up the buffer (why?) but notes to not disturb the buffer. What is this buffer being used for? 4.5: This is unclear. Not sure what these instructions mean, a video may help.

Response: We thank the reviewer for all the questions regarding the mounting procedures from Step 4.2 to 4.5.

We agree with the reviewer that without the help of video, it is really hard to explain explicitly and to make sense of each exact step. We believe it will make more sense when the writing is accompanied with the video that we will prepare with help of the JoVE staff.

6.1: Imaging is one of the areas that differs between laboratories and leads to variation in data. It should be added here that imaging parameters should be kept the same.

Response: We thank the reviewer for all the questions.

We've elaborated the procedures of imaging in the revised manuscript:

"Imaging

6.1. Turn on the scanner and the computer (see Table of Materials). Position the slides in the slide holder with the loading device and insert the holder into the scanner.

Note: The scanner enables scanning of multiple channels (e.g., DAPI, green and red channel) simultaneously. The scanner scans slides only with a 20X magnification, and the Leica microscope (see Table of Materials) can be employed when a higher magnification (e.g., 40X) is needed.

6.2 Open the ZEN 3.1 software for the scanner. Choose the appropriate storage location and scanning profile.

6.3 Start the preview scan by clicking the "Start Preview Scan" button. After preview scan, open the tissue detection wizard and circle the regions of interest you want to image.

6.4 Click the "Start Scan" button after choosing your regions to image. Wait for the machine to finish scanning.

6.4 Check the result file and export the images.

Note: If the profile is not suitable for the slide, adjust by refocusing, changing the exposure time or light strength to adapt the profile to your tissues. Refer to the instruction of Zeiss Axio Z1 Scanner for more technical details." (Line 257-274)

7.2: This grammatically incorrect and should be rewritten. 7.3: How many slices per animal, just 1? Also, 3 animals per group is not sufficient for statistical analyses unless there are multiple data points (sections) per animal, and still more than 3 animals are usually needed. It is better to say a power calculation should be done to determine the number of animals needed. If there is a neuron population that is present in one group and absent in another group, then 3 animals would show a difference. If there is an injury-induced change in neurons that is subtle, then 10 animals a group might be needed to see a difference.

Response: We thank the reviewer for all the questions.

Since data analysis is not the focus of this protocol, we decided to delete it in the revised manuscript.

Discussion:
Doesn't make sense, 'The purpose of perfusion is to flush out blood thoroughly from the brain, thereafter fix the brain'.
There are published papers (in JOVE) that show you can cut multiple brains at once that are embedded in OCT so the statement about sucrose being better is not accurate for the reasons listed. This section should be removed.

Response: We thank the reviewer for the question.

We have re-written it in the revised manuscript as follows:

"The quality of perfusion is critical for the successful staining thereafter" (Line 317)

We have removed the statement that sucrose is more advantageous than O.C.T. from the perspective of economic option in the revised manuscript.

More details need to be added about the cocktail of primary antibodies. Specifically, add information about cross reactivity.

Response: We thank the reviewer for the question.

We have provided more details about the cocktail of primary antibodies and guideline of selection of primary, secondary and blocking serum in the revised manuscript:

"A cocktail of primary antibodies and secondary antibodies might be used in the context of double-staining to speed up the procedure. More specifically, two different primary antibodies from different species (e.g., one is from rabbit, the other one is from guinea pig, chicken or mouse) are mixed together before incubation, as are the corresponding secondary antibodies. The choice of secondary antibody is dependent on primary antibody. If the primary antibody is from rabbit, then the secondary antibody must be anti-rabbit, for example donkey anti-rabbit or goat anti-rabbit. While the selection of blocking serum depends on the secondary antibody, for example donkey normal serum will be used if the secondary antibody is from donkey. Antigen retrieval is suggested if the immunostaining still does not work even if all guidelines have been followed strictly." (Line 353-362)

There is no data to support that this cryosectioning strategy is much more efficient compared to the conventional practice. This is an overstatement with no support and should be removed.

Response: We thank the reviewer for the comment.

For conventional cryosectioning, which has been extensively used nowadays, researchers can only cut one brain sample each time, while our microtome strategy allows researchers to cut multiple brain samples (5 or 6) each time. This would save quite a lot of time for researchers, and largely improve efficiency.

In fact, we have many years' experience working with conventional cryosectioning. We shifted to this microtome strategy as the conventional one is not efficient enough, particularly when we have many brain samples for sectioning. Therefore, we would like to keep it in the revised manuscript.

The statement that a regular microscope cannot cover the whole brain section with an appropriate magnification is unclear and not accurate and should be removed.

Response: We thank the reviewer for the comment.

We have removed it from the revised manuscript.