Video Article

Simultaneous Cryosectioning of Multiple Rodent Brains

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Abstract

Histology and immunohistochemistry are routine methods of analysis to visualize microscopic anatomy and localize proteins within biological tissue. In neuroscience, as well as a plethora of other scientific fields, these techniques are used. Immunohistochemistry can be done on slide mounted tissue or free-floating sections. Preparing slide-mounted samples is a time intensive process. The following protocol for a technique, called the Megabrain, reduced the time taken to cryosection and mount brain tissue by up to 90% by combining multiple brains into a single frozen block. Furthermore, this technique reduced variability seen between staining rounds, in a large histochemical study. The current technique has been optimized for using rodent brain tissue in downstream immunohistochemical analyses; however, it can be applied to different scientific fields that use cryosectioning.

Video Link

The video component of this article can be found at https://www.jove.com/video/58513/

Introduction

Here, we present the protocol for a novel method, which we call Megabrain, developed to cryosection multiple rodent brains simultaneously for downstream immunohistochemical procedures. A Megabrain allows for the production of single slides containing tissue from multiple animals. This technique has been optimized to cut coronal sections from 9 adult rat hemispheres, or 5 adult full brains, simultaneously. Therefore, the technique is most applicable in large immunohistochemical studies or other analyses done on slide-mounted brain tissue from a large cohort of animals.

Immunohistochemistry involves the use of specific antibodies directed against proteins of interest to understand and characterize their expression and cellular changes in specific tissue[1,2,3]. The use of immunohistochemistry is prevalent in neuroscience research, among other scientific disciplines, aiding in the cellular and molecular understanding of the brain[4]. Large-scale studies involving many animals and brain sections can be both resource and time intensive. As such, there is a multifaceted rationale behind the development of the Megabrain: to reduce time spent cryosectioning, mounting, and staining tissue, while consequently using less reagents. Moreover, the ability to streamline the process and stain multiple brains in the same round helps to alleviate some of the variability between staining batches, a limitation of immunohistochemistry[3]. In addition, sectioning a Megabrain is a time saving alternative to sectioning individual frozen rodent brains and allows for rapid comparison of tissue between animals or even treatment groups by microscopy techniques.

Protocol

The Megabrain technique has been optimized using whole and hemisected brains from male adult C57Bl6 mice[5], juvenile Sprague-Dawley rats, and adult Sprague-Dawley rats that were transcardially perfused with 1x phosphate buffered saline (PBS) followed by 4% paraformaldehyde[6]. Similar outcomes can be accomplished in other strains of mouse and rat, in both sexes and at different ages. The study to generate data for this manuscript used juvenile Sprague-Dawley rats and was approved by the University of Arizona Institutional Animal Care and Use Committee, and experimental animals were cared for according the Guide For the Care and Use of Laboratory Animals[7].

1. Cryoprotection of Perfused Brains

1. Following successful transcardial perfusion[6], collect the full brain[6,8] from animals and post-fix by placing into a 25 mL vial of 4% paraformaldehyde in PBS for 24 h.
   CAUTION: Paraformaldehyde is a toxic tissue fixative; handle with care. Transfer paraformaldehyde into a specifically labeled waste container that identifies its concentration and volume inside a chemical fume hood and then store in the chemical waste cabinet until disposed by Chemical Safety or properly trained personnel.
2. After 24 h has elapsed, in a fume hood, remove brains from 4% paraformaldehyde using a spatula. Transfer the brain into a vial of approximately 20 mL of 15% sucrose solution in 1x Tris-buffered saline (TBS) until brain sinks to the bottom of the vial (approximately 24 h).
3. Following this, transfer the brain to a vial of approximately 20 mL of 30% sucrose solution in 1x TBS, until the brain sinks to the bottom of the vial (approximately 24–48 h).

2. Brain Freezing

1. Place a 500 mL glass beaker on a bed of dry ice in an ice bucket. Pour 300–400 mL of isopentane (2 methyl-butane) into the glass beaker.
2. Allow the temperature of the isopentane to reach between -45 °C and -50 °C. Closely monitor and maintain this temperature range with a thermometer, keeping it constant throughout the procedure. Ensure that temperature readings are taken from the middle of the solution, and not while touching the bottom or side of the beaker.
3. On the benchtop, fill a disposable embedding mold (see materials list) approximately 1/2 full of Optimal Cutting Temperature (OCT; see **Table of Materials**) Compound. Ensure that there are no air bubbles in the OCT. If there are any air bubbles remove them with a spatula or needle.
4. **Remove the first brain from the vial of 30% sucrose with a spatula. At this point, either freeze brains whole or hemisect, depending on the study design.**
   1. For hemisected brains, use a new razor blade to make a clean cut through the tissue to separate the hemispheres. If not required for the study, remove the cerebellum and olfactory bulbs at this stage. If the cerebellum is needed for the study, it is suggested to cut a flat area at the posterior end of the brain to aid the brain in standing straight in the mold. If the study only requires tissue from a localized region, a rodent brain matrix can be used to block the brain based on known coordinates.
5. Give brains a numeric naming system. Draw a diagram as shown in **Figure 1**. Write the number of the first brain to be placed in the Megabrain mold in position 2, leaving position 1 as a blank space to aid in quick identification of the orientation in which the brains were frozen.
6. Using blunt forceps or tweezers, pick up the brain to be placed in position 1. Orient the brain with the side to be cut first facing upwards. Lower the brain into the OCT in the appropriate location, using the tweezers to adjust its position until it stands independently.
7. Repeat steps 2.3–2.5 for the remaining brains/hemispheres to be frozen in positions 3–10. Avoid positioning brains/hemispheres in a symmetrical layout.
8. Review all 9 brains in the Megabrain mold. Once the brains are standing independently, upright, and correctly oriented in the OCT (as shown in **Figure 2B**), add more OCT to the mold (until the top of the brains are covered). Again, ensure that there are no visible air bubbles in the OCT.
9. Use the forceps to hold the corner of the mold, ensuring the forceps do not become partially submerged in the OCT (**Figure 3A**). Being careful to keep the mold level, lower the mold into the isopentane (-45 °C to -50°C) so that the bottom third of the mold is submerged. Hold it here to allow any bubbles in the OCT to rise to the surface and away from the tissue (**Figure 3B**).
10. After 30 s, lower the entire mold into the isopentane and release from the forceps, leaving the Megabrain submerged for at least 3 min (**Figure 3C**). Ensure that mold is kept level, so brains remain upright and equidistant (**Figure 3D**).
11. After 3 min, use the forceps to remove the mold containing the frozen, OCT embedded brains (**Figure 3E**) from the isopentane.
12. Immediately, wrap the mold and its contents in aluminum foil and label it with the animal numbers or Megabrain identification. Touch the frozen Megabrain as sparingly as possible to avoid thawing the tissue.
13. This Megabrain can now be transferred to a -20 °C freezer and stored for a minimum of 24 h.
    NOTE: Protocol can be paused here and the Megabrain can be stored frozen until cryosectioning takes place.

3. Preparing the Megabrain for Sectioning on the Cryostat

1. Set the cabinet temperature of the cryostat to -19 °C. Make sure this temperature is reached before continuing. Throughout sectioning, ensure that the cabinet temperature remains between -18 °C and -20 °C.
2. Take the Megabrain from the -20 °C freezer and place it into the cryostat. Also, place a chuck of the appropriate size dimensions, and two thin tipped paintbrushes into the cryostat. Leave both the Megabrain and the chuck in the cryostat for at least 15 min to come to the temperature of the cryostat.
3. Appropriately label slides with the Megabrain identification number and the slide number. Lay these slides on a slide warmer (35–45 °C).
4. Unwrap the Megabrain from the aluminum foil. Use a razor blade to vertically cut the corners of the mold to allow easy removal of the block of OCT from the mold (**Figure 4A**).
5. Dispense a thin layer (approximately 3 mm thick) of OCT onto the chuck.
6. Quickly, with minimal contact between fingers and the OCT, place the Megabrain on the chuck, in the orientation desired. Large forceps can also be used for this step to minimize thawing. Before starting to section, leave the Megabrain mounted chuck in the cryostat for 15–20 min to allow the OCT to freeze completely.
7. Once the base layer of OCT has frozen, apply a 2 mm thick layer OCT around the sides of the Megabrain and allow this to run down from the sides onto the chuck. This helps secure the Megabrain to the chuck. Again, before continuing, leave the Megabrain mounted chuck in the cryostat for 15–20 min to allow the OCT to freeze.
8. Use a razor blade to remove any excess OCT from the sides or bottom of the chuck (**Figure 4B**).

4. Cryosectioning the Megabrain

1. Before starting, ensure that a beaker containing at least 20 mL 1x PBS is accessible.
2. Position the chuck, mounted with the Megabrain, into the chuck head on the cryostat (**Figure 4B**).
3. Set the cryostat to cut at the desired thickness. The current methodology has been optimized for 10 µm to 50 µm sections.
4. Trim the OCT and tissue as needed to reach the desired brain region for tissue collection (**Figure 4C**).
5. When ready to collect tissue, lower the anti-roll plate until it rests on the stage and turn the cryostat handle to take a single section. Slowly lift off the anti-roll plate, being careful that the sectioned Megabrain is not attached to the anti-roll plate and does not fall off the stage **Figure 4D**).
6. Gently use the paintbrushes to unroll the Megabrain section until it is lying flat on the cryostat stage (**Figure 5A**). (If needed, hold the OCT flat using the paintbrushes to prevent rolling).
7. Remove slide from slide warmer and hover the slide, label-side down, over the Megabrain section on the stage allowing the section to stick to the slide.
8. Quickly apply a drop of 1x PBS to each tissue section on the slide and do not allow these to dry out until they have been brushed flat (see step 4.9).
9. Use a fine tipped paintbrush dipped in 1x PBS to brush out any bubbles in the tissue and unfold the tissue so it is lying flat on the slide (**Figure 5B**). Take care not to change the position of the brain sections and thus lose the relative position to the other tissue sections. Manipulate the tissue with care to avoid tears. Keep brain sections away from the edges of the slide, as peripheral space is required for a coverslip and PAP pen application in some staining protocols.
10. Place the slide back onto the slide warmer and dry for 45 min. Cover as needed to prevent dust from settling on the tissue. Once dry, store the slides in at -80 °C until further use.

Representative Results

A positive end result to this procedure is tissue that lies flat on the slide, with no bubbles or tears, in the orientation in which they were frozen. Tissue sections are evenly spaced apart and easily identifiable due to good placement of the brain in the OCT and good notation as demonstrated in **Figure 1**. Assuming that the brain tissue was collected from animals of a similar age, and that the tissue was properly aligned in the OCT, sections collected on a slide should represent a similar coronal plane, allowing comparison of brain regions between animals. Provided that the tissue has frozen well, with minimal freeze artifact, H&E stain can be carried out to assess the cryoprotection quality[9]. Immunohistochemistry can be carried out as demonstrated with the staining shown in **Figure 6**.

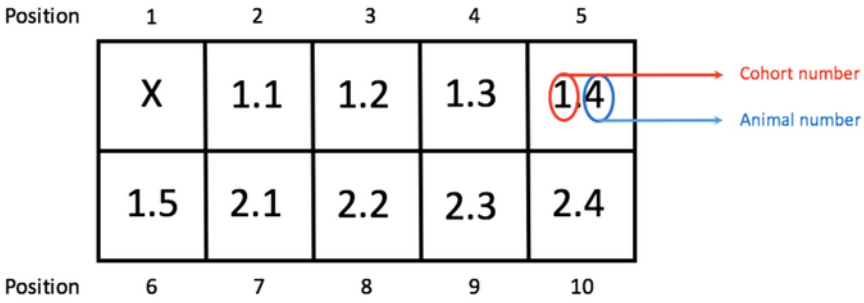

Figure 1: Representative diagram of a suggested Megabrain layout. This shows the position notation of tissue derived from 9 different animals. The number system is designed as demonstrated; however, any numbering system may be used. 'X' is representative of the blank space designed to clearly show the orientation in which the tissue was frozen. Please click here to view a larger version of this figure.

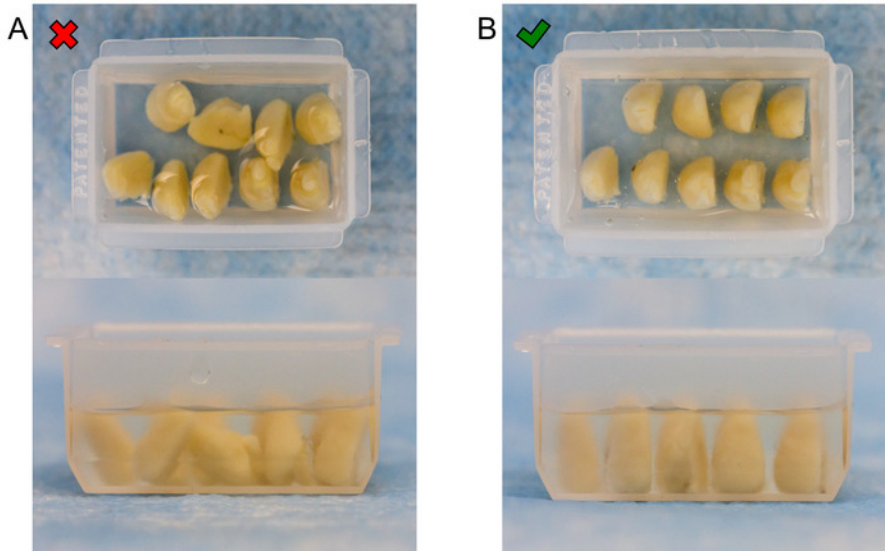

Figure 2: Incorrectly and correctly positioned brains in mold. (**A**) Brain hemispheres poorly aligned in OCT from the top and side. (**B**) Well-aligned brain hemispheres in OCT from the top and side. Please click here to view a larger version of this figure.

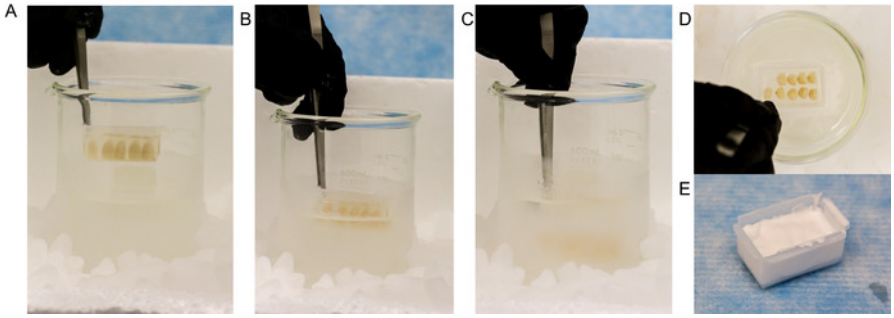

Figure 3: Stages of brain freezing. (**A**) Megabrain before being submerged in -45 °C isopentane. (**B**) Megabrain when submerged in isopentane. (**C**) Megabrain fully submerged in isopentane. (**D**) Megabrain being lowered into isopentane, demonstrating that brains should stay upright and equidistant from one another during this process. (**E**) A frozen Megabrain in an embedding mold. Please click here to view a larger version of this figure.

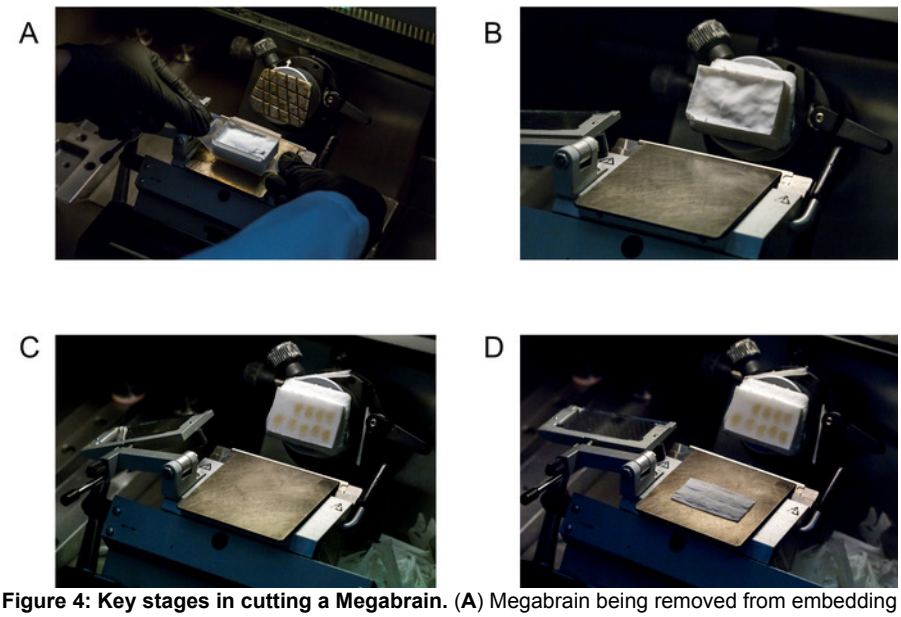

Figure 4: Key stages in cutting a Megabrain. (**A**) Megabrain being removed from embedding mold in cryostat. (**B**) Chuck mounted with Megabrain. (**C**) Trimmed Megabrain. (**D**) Megabrain section on cryostat stage. Please click here to view a larger version of this figure.

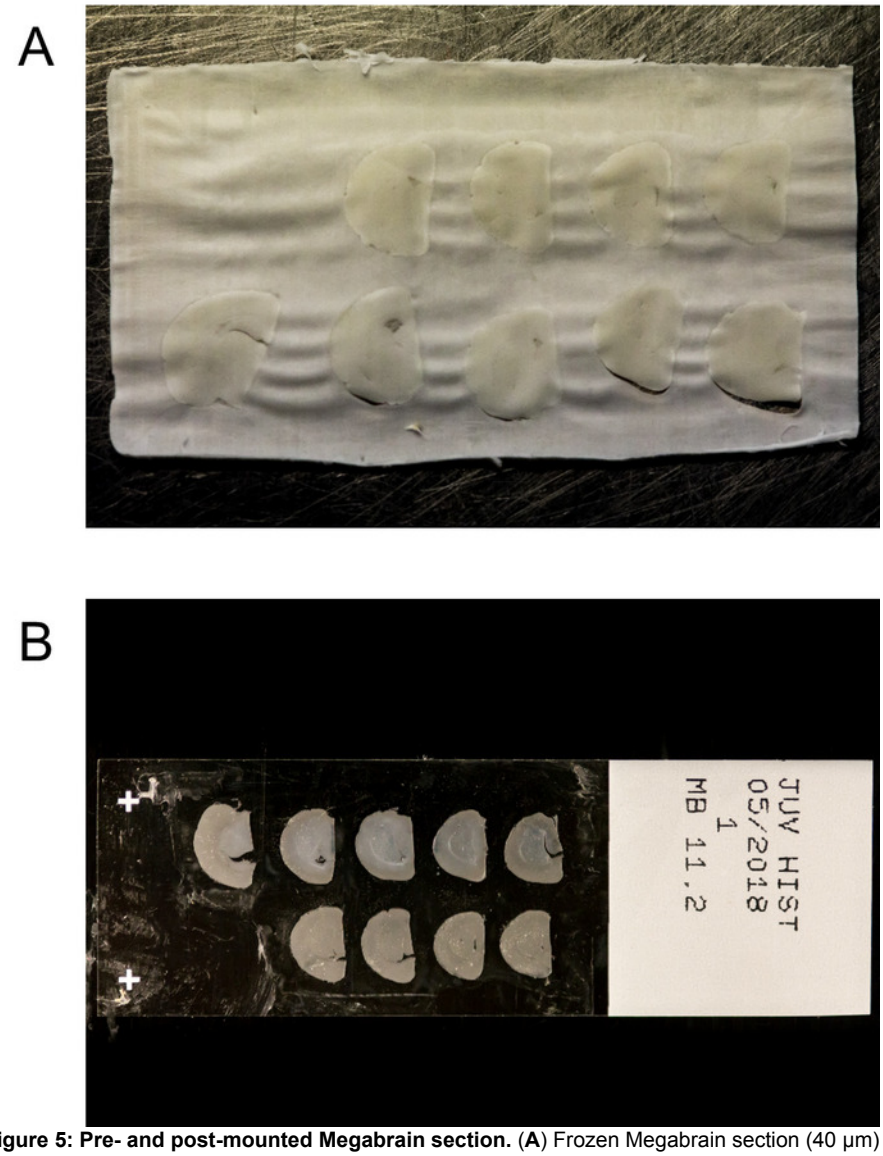

Figure 5: Pre- and post-mounted Megabrain section. (**A**) Frozen Megabrain section (40 µm) on cryostat stage. (**B**) Glass slide mounted with Megabrain tissue. Please click here to view a larger version of this figure.

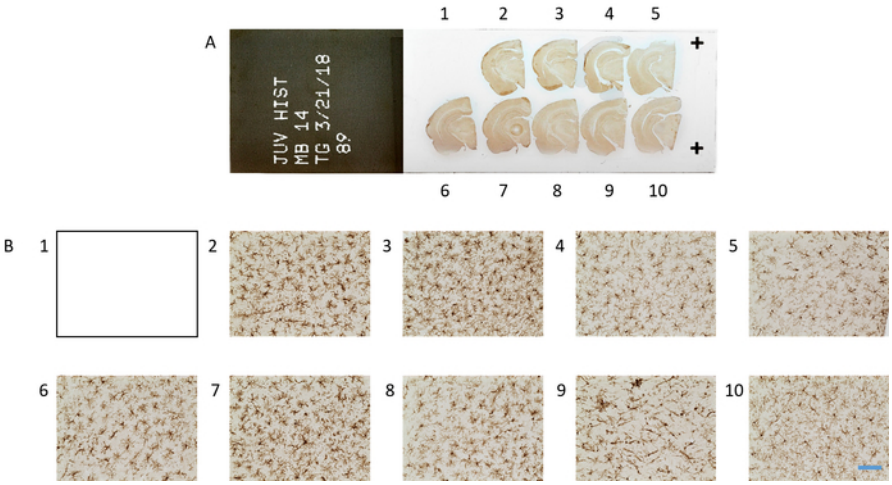

Figure 6: Immunohistochemistry of a Megabrain slide stained with Iba1. (**A**) Tissue taken from a Megbrain shows uniform staining between different brains in a study. (**B**) 20x microscopic images taken from the somatosensory barrel field (S1BF) cortex region of each mounted brain, showing consistent ionized calcium-binding adapter molecule 1 (Iba1) staining of microglia between brains. Scale bar = 100 µm. Please click here to view a larger version of this figure.

Discussion

It should be considered during this procedure that the temperature of the Megabrain and its surroundings must be constantly monitored to prevent thawing and re-freezing of the tissue. The brain can only be removed from the -20 °C freezer up to 3 times as every time it is touched and left in the cryostat, with a warmer fluctuating temperature, the tissue thaws and refreezes, causing a jelly like texture and abnormal tissue integrity[10]. Therefore, it is optimal to cut the Megabrain all at once.

Tissue fixation and cryoprotection are key parts of this protocol to minimize ice crystal formation, reduce external microbial growth, and stop enzymatic reactions, thereby preserving tissue integrity[10]. If the brains are not cryoprotected with sucrose, then water within the tissue can form ice crystals during the freezing process, which shred the tissue and cause holes to form. While we suggest serial dilutions of sucrose in TBS, brains can be placed directly in 30% sucrose for an extended period (48-72 hours) to achieve similar cryoprotection. However, it is highly recommended that the sucrose solutions are made with TBS. Variations such as the use of PBS or water will result in poor cryoprotection of the Megabrain and poor tissue quality. Haematoxylin and Eosin (H&E) stain can be used to assess quality of the cryoprotection and consequent freeze artifact[11]. Good tissue quality (with minimal holes) is essential in histological studies as the holes can disrupt the binding of antibodies as well as reducing the tissue integrity of cells with fine processes, which can affect the analysis of some stains. Avoid symmetrical placement of brains, as this can cause confusion when trying to identify the animal from which each brain was obtained.

The current technique has been optimized for coronal sections; however, it can easily be adapted to cut sagittal and transverse sections. These cutting modifications will require appropriate variation of brain placement in steps 2.5 through 2.7. It should be noted that these adaptions may reduce the number of brains that can fit into one embedding mold.

A key modification of this technique is the blank space at position number 1 (as shown in **Figure 1**). This space is designed to allow easy identification of the Megabrain orientation, and therefore identify the animal associated with each brain.

This technique has been modified to allow tissue from a larger number of animals to be frozen and cut simultaneously in the Megabrain. The size of the rodent brain is dependent on sex, age and species therefore; the number of brains that fit in the mold can vary. It is not advised to segregate different ages, sexes, or treatment groups into separate Megabrains. Although this can ensure size uniformity, it may also increase bias and subjectivity during imaging and analysis. In addition, there is inevitable variance between slides during immunohistochemistry in any protocol, which could lead to staining inconsistency between age/sex groups.

A rectangular, larger chuck was used, in place of a circular one, which provided increased support behind the Megabrain.

As the mold and its contents were of a larger volume than that of a singular brain in an OCT mold, more time was required to freeze the brain in the isopentane. Through trial and error, a length of time that provided good freeze quality (3.5 minutes) was determined. The Megabrain can be left in the isopentane for longer; however, the temperature must stay in the range of -45 °C to -50°C to avoid cracking the OCT.

As there are many separate pieces of tissue on the Megabrain slide, it is possible that tissue can dry out and consequently become more brittle during the brushing steps. To combat this issue, a drop of PBS should be put on each individual brain section to keep it moist until it is brushed out.

The technique of hemisecting the brain, for a study in which this is required, is critical to obtaining good results. When hemisecting the brain, it is important that this bisection is done precisely to ensure all brain regions are intact with the correct hemisphere. If the cerebellum or olfactory bulbs are not relevant to the study, they can be removed. Removing this 'excess' tissue reduces the amount of tissue that will need to be trimmed when cryosectioning. Removing the cerebellum allows the brains to stand up more easily in the OCT before freezing.

While being brushed out, keep the tissue sections away from the edge of the slide. This allows room for a line of PAP pen, a hydrophobic barrier, to surround the tissue, which is a requirement of some immunohistochemistry protocols. Additionally, space is needed for the coverslip.

If the hemisphere/brain falls or tilts during the Megabrain freezing, then the sections will not be co-planer. A similar outcome can result if one brain is larger than the others in a single Megabrain. To overcome this, more slides can be selected to stain where the brain(s) in question are at the appropriate area of interest.

One limitation of this technique is that as all of the tissue is frozen in one block of OCT; if there is technical error, such as a bad freeze due to an incorrect temperature, then the tissue from multiple animals will be affected (5-9 animal brains instead of just 1). This is more of a problem when using the full brain and not hemisected sections, because the remaining hemisphere can be frozen and sectioned singly to obtain the desired sections.

Another limitation to this method is that when adjusting the cryostat to cut the Megabrain, a best-fit approach must be taken. This is due to all brains being at slightly different angles to one another, whereas this is not an issue when sectioning a singularly frozen brain. The effects of this can be minimized during freezing by ensuring brains are upright and of a similar size.

One of the biggest benefits of using this technique over freezing brains singularly is that it shortens the overall time spent freezing and cutting brains by up to 90%, if 9 are being cut at the same pace as 1 brain. The other key advantage of this technique is that it allows a smaller number of slides that contain tissue from the required animals to be stained. This increases the reliability and uniformity of immunohistochemical staining, since all slides are stained in the same round. There can be inconsistency between slides stained at the same time, but a Megabrain can allow several study brains to be represented on a single slide, thus reducing variability and increasing the validity of the analysis. Having consistent staining is a key advantage in any histological study and a major significance of using this technique. Lastly, fewer slides also incur lower supply costs.

This technique could be easily modified for cutting sagittal sections of a rodent brain. It could also be adapted for use in cryosectioning the tissue from different animal models and different types of tissue, such as muscle or liver samples. Adaptions to the protocol would have to be made, including optimization of the amount of solutions used, size of the cryostat blade/stage, and the volume of the embedding mold.

This technique could also be adapted for free-floating techniques, where the protocol would remain the same until step 4.6. At this step, brains would need to be separated and lifted into separate plate wells to be stained. Extra care would need to be taken not to confuse the brain slices in the sorting process.

Disclosures

The authors have no disclosures.

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