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Collection of Frozen Rodent Brain Regions for Downstream Analyses

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TITLE:
Collection of Frozen Rodent Brain Regions for Downstream Analyses

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KEYWORDS:
Medial prefrontal cortex, dorsal hippocampus, caudate putamen, nucleus accumbens, brain matrix, biopsy punch.

SUMMARY:
This procedure describes the collection of discrete frozen brain regions to obtain high-quality protein and RNA using inexpensive and commonly available tools.

ABSTRACT:
As our understanding of neurobiology has progressed, molecular analyses are often performed on small brain areas such as the medial prefrontal cortex (mPFC) or nucleus accumbens. The challenge in this work is to dissect the correct area while preserving the microenvironment to be examined. In this paper, we describe a simple, low-cost method using resources readily available in most labs. This method preserves nucleic acid and proteins by keeping the tissue frozen throughout the process. Brains are cut into 0.5–1.0 mm sections using a brain matrix and arranged on a frozen glass plate. Landmarks within each section are compared to a reference, such as the Allen Mouse Brain Atlas, and regions are dissected using a cold scalpel or biopsy punch. Tissue is then stored at -80 °C until use. Through this process rat and mouse mPFC, nucleus accumbens, dorsal and ventral hippocampus and other regions have been successfully analyzed using qRT-PCR and Western assays. This method is limited to brain regions that can be identified by clear landmarks.

INTRODUCTION:

This work illustrates the dissection of frozen brain regions for extraction of high-quality nucleic acid or protein using a reference, such as the Allen Mouse Brain Atlas[1], as a guide. In this technique, brains are flash-frozen and stored at -80 °C for later sectioning and dissection while being maintained in a frozen condition. This process allows the researcher to harvest a large number of brains in one session and later dissect them for an accurate collection of multiple brain regions.

The accurate collection of brain regions of interest (ROIs) is often required when answering questions related to gene and protein expression. While pharmacology, electrophysiology and optogenetics can be used on wildtype or genetically modified rodents to help elucidate molecular changes underpinning observed behaviors[2-4], the measurement of induced changes in transcriptomes and proteomes is often used to support these findings. Techniques such as quantitative reverse transcription polymerase chain reaction (RT-qPCR), western blotting, RNAseq[5], MAPSeq[6] and HPLC[7] are robust and relatively low in cost, allowing many labs to study induced molecular changes within small brain regions[2,4-6].

There are several ways to extract and purify nucleic acid or protein from brain regions[8-12]. Many labs harvest brain regions by chilling and cutting brains on ice at the time of harvest[9,13]. While this approach can result in high quality nucleic acid and protein, it is somewhat time-limited as degradation within the microenvironment of the tissue may take place at these temperatures. This may be particularly true when attempting to dissect a large number of animals or ROIs in one sitting. Keeping samples frozen helps maintain labile target molecules while providing the researcher time to carefully compare landmarks on both sides of each section in the effort to collect relatively pure samples. Laser capture is another way to collect tissue for RNA or protein analysis from brain areas[10]. This procedure is superior to mechanical dissection in that very small and irregularly shaped ROIs can be identified and isolated. However, laser capture is limited by the use of expensive equipment and reagents, is time consuming and may also be more susceptible to sample degradation.

Micropunch dissection on frozen tissues is not new. Early papers by Miklos Palkovits and others describe the basic techniques in detail[14,15]. This presentation largely follows the original work, with some improvements to facilitate efficiency and decrease the expense of the equipment needed. For instance, brain sections are made in a frozen brain block rather than on a cryostat. This produces thicker sections which reduces the number of sections needed to collect ROI samples. This method also dissects samples on a frozen glass plate that sits on dry ice within an insulated box. This produces a sub-freezing stage at the bench on which to work. Sections dissected in this way are easily manipulatable, allowing the researcher to compare both sides of each section with a reference in order to limit contamination from regions outside the desired ROI.

Advantages of this protocol are that 1) the brain is kept in a frozen condition throughout the process, which helps preserve protein and nucleic acid and gives the researcher time to carefully harvest ROIs, and 2) the reagents required are inexpensive and are found in most molecular

biology labs. In this process, whole brains are sectioned to 0.5–1.0 mm in a brain matrix and placed on a frozen glass plate that is continuously chilled with dry ice. Landmarks found in the Allen Brain Atlas[1] or other brain atlases[16,17] are used to identify regions of interest, which are then dissected using either a cold punch or scalpel. Because the tissue is never thawed, regions harvested in this manner provide high quality RNA and protein for downstream analyses.

PROTOCOL:

Animals used in this study were treated in an ethical and humane manner as set forth by Indiana University's Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH) guidelines.

NOTE: All tools and surfaces should be washed with an appropriate solvent to remove nucleases[18] before starting any work.

1. Storing brains

1.1. Quickly remove the brains from euthanized adult CD1 wildtype mice weighing approximately 30 g using a conventional approach[13] and flash-freeze for 60 seconds in either liquid nitrogen or isopentane pre-chilled with dry ice.

1.2. Store frozen brains at -80 °C in aluminum foil or conical tubes until use.

2. Preparing the brain matrix

2.1. Twenty-four hours before dissecting tissue, place a clean brain matrix (see **Table of Materials**) on a stack of thawed freezer packs. Sandwich the sides of the matrix between two freezer packs making sure to leave approximately 0.5 cm between the bottom of the razor slots and the top of the gel packs (**Figure 1A**). Place aluminum foil on the ends to aid in cooling.

NOTE: When purchasing a brain matrix, buy one that is large enough to encase the entire volume of the brain to be dissected.

2.2 Place the box containing the brain matrix and freezer packs in a -20 °C freezer with the top ajar overnight.

3. Setting up a frozen glass plate

NOTE: The purpose of this setup is to prepare a frozen surface on which to dissect brain sections.

3.1. Place ice in an insulated box up to approximately 5 cm from the top. Then place a 2.5 cm layer of dry ice on top of the ice and cover with black plastic sheeting to aid in visualizing the sample (**Figure 1B, Figure 1C**).

3.2. Place a glass plate (should just fit in the opening of the box) on top of the plastic and place dry ice on top of the plate in the far corners (**Figure 1C**).

3.3. Take the frozen brain matrix from the freezer and insert the brain to be cut cortex-side up. Allow it to equilibrate to the temperature in the box for 10 min. Keep the lid on the box during this time.

3.4. Adjust the brain's position in the matrix with cold forceps so that the sagittal sinus and transverse sinus line up with the perpendicular grooves of the block (**Figure 1D**). This will help ensure symmetric sections for easier dissection. Touch tips of forceps to dry ice briefly to chill before adjusting the brain.

3.5. Once the brain is in position, place a chilled razor blade near its center and press the blade approximately 1 mm into the tissue. Add chilled blades to the rostral and caudal ends to help hold the brain in place (**Figure 2A** and **Figure 2B**).

3.6. Starting on the rostral end, add blades one at a time, placing them into the slots and pressing them gently down into the tissue approximately 1 mm (**Figure 2B**). Continue to add blades at 1 mm intervals working towards the caudal end.

3.7. Make sure the brain does not shift during this time. Line up blades horizontally and vertically (**Figure 2B**). In order to cut sections into 0.5 mm widths, low profile microtome blades (see **Table of Materials**) may be used. Place these between the larger razor blades (**Figure 2C**).

3.8. Once blades are in place, press down on top of the group with fingers, palm or some other flat surface (**Figure 2C, Figure 2D**). Rock the group of blades slowly from side to side to move them through the tissue.

NOTE: This may take some time (1–2 min) and requires patience. There should be resistance to the blades moving through the tissue. Easy entry means the brain is thawing and the block should be cooled with dry ice.

3.9. When all blades have reached the bottom of the slots, grasp each side of the group of blades and work free of the matrix by rocking back and forth.

NOTE: Exercise caution to avoid cutting oneself on the sharp edges of the blades.

3.10. Once the group is free, place the stack, rostral side up, on the glass plate (**Figure 2E**). Place dry ice next to and/or on top of the stack to further freeze the samples for easier separation.

3.11. Place the stack with sharp edges down on the glass plate and separate blades by shifting the stack between thumbs and fingers. The blades should separate from each other with sections attached.

3.10 Line up sections on the glass plate from rostral to caudal (**Figure 2F**).

3.11 Separate tissue from the blades by flexing blades between fingers, or by separating with a second cold razor (**Figures 2G,2H,2I**).

4. Dissecting sections

4.1. Open the Allen Mouse Brain Atlas or another reference and find landmarks necessary to identify regions of interest. Some obvious landmarks include the anterior commissure, the corpus callosum, the lateral ventricles, and the hippocampus (**Figure 3**).

4.2. Flip the section to be cut with chilled forceps and make sure the region about to be collected is consistent throughout the section. During harvesting, consult the reference atlas often to make sure the correct ROI is obtained.

NOTE: A magnifying glass or dissecting microscope is often helpful in this process. Good lighting is also essential. Low wattage LED lamps or a cool lamp (see **Table of Materials**) can be used for this purpose.

4.3. With a clean scalpel or punch, cut into the section (**Figure 4**). Prechill each tool before cutting by touching it briefly to dry ice. Push the blade gently but firmly into the tissue, rocking it back and forth to make the cut. Do not push too hard or the tissue will fracture.

NOTE: Tools will warm over time. Slightly warmed blades can be helpful in making clean cuts and can limit fracturing, but be careful to avoid thawing of tissue. Periodically chill tools on dry ice.

4.4. Once an ROI is harvested, place it into labeled, prechilled 1.5 mL tubes. Store harvested tissues at -80 °C until needed.

4.5. Process frozen tissue collected in this manner by adding cold RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% CHAPS and protein inhibitor cocktail (see **Table of Materials**)) for protein extraction, or a guanidinium-containing solvent (see **Table of Materials**) for RNA extraction to the frozen sample and immediately homogenize using either a glass Dounce or mechanical homogenizer (see **Table of Materials**). In this way, protease and nuclease inhibitors are in place as the sample warms to protect protein and nucleic acids from degradation.

REPRESENTATIVE RESULTS:

In order to validate this method, the medial prefrontal cortex was collected from adult CD1 wildtype male mice and RNA and protein were extracted and characterized. RNA was analyzed by capillary electrophoresis. Degraded RNA displays a loss in the intensity of the 28S and 18S ribosomal bands and also shows degradation products as a smear between 25 and 200 nucleotides (**Figure 5A**, sample 1). High quality RNA shows distinct ribosomal bands with little to no signal in the lower molecular weight region (**Figure 5A**, sample 2). RNA integrity numbers

(RINs) are also generated in this analysis. RINs above 7 indicate good quality RNA[19]. RNA isolated from samples using the frozen dissection method display strong ribosomal banding and high RIN values comparing very favorably with RNA prepared from freshly harvested tissue (**Figure 5B**).

One advantage of this method is that a large number of brains can be quickly harvested and stored in one sitting to be carefully dissected at a later time. ROIs can also be stored without the tissues being thawed. With this method, a researcher can collect a large and diverse collection of ROIs from which high-quality nucleic acid or protein may be obtained. To confirm this point, RNA was extracted from the dissected mPFC that had been stored over several weeks (harvested brains had been stored for several months). All samples produced high quality RNA with distinct ribosomal banding and RINs above 8 (**Figure 5C**).

Western blot analysis was used to confirm protein integrity of frozen dissected mPFC as previously described[20]. Western blots display reduced signal of high molecular weight targets when protein is degraded. The degradation products also show up as a smear at lower molecular weights within the column. For this analysis protein was collected, transferred to nitrocellulose, and probed with antibodies against a high molecular weight target, KCC2, and the lower molecular weight protein, actin (**Figure 5D**). In all cases, bands were sharp and distinct with no discernable breakdown products for either KCC2 or actin. These experiments confirm that the dissection of ROIs using this method enables the extraction of high-quality RNA and protein.

FIGURE LEGENDS:

Figure 1: Preparing frozen work surfaces. (**A**) A brain block is placed on a stack of gel packs within an insulated box and is then sandwiched between two additional gel packs. Aluminum foil is packed at each end of the block to facilitate cooling during sectioning. The box is then cooled overnight at -20 °C. (**B**) A glass plate is matched for size with the opening of an insulated box. A shipping box can be used for this purpose. Ice is added to the box until it is approximately 5 cm from the top. The box is then frozen overnight at -20 °C. (**B, C**) Just before collecting tissue, a uniform layer of dry ice is placed on top of the ice at a depth of approximately 2.5 cm. A sheet of black plastic (to aid in visualization) is placed over the dry ice followed by the glass plate. Dry ice is placed in the far corners, which cools the air just above the plate. Tools can be cooled by laying them on the plate, or by briefly touching them to the dry ice. (**D**) To ensure symmetric sections, the brain should be positioned with cold forceps so that the sagittal sinus and transverse sinus line up with the perpendicular grooves of the block (white arrows).

Figure 2: Sectioning brain using a brain block. (**A**) The orientation of the brain is anchored by seating conventional single edge razor blades into the cortex at a depth of approximately 1 mm. Placing one in the middle and one at each end helps to hold the brain in position. (**B**) Add blades one at a time until all slots are filled. (**C**) Low profile blades can be inserted between conventional blades when 0.5 mm sections are desired (red arrow). (**C, D**) The group of blades is pushed into the frozen tissue with moderate downward pressure, using either a flat instrument or the palm or fingers of the hand. Blades can be slowly rocked back and forth to help move them through the tissue. (**E**) Sections are removed by grasping the sides of the razor blades and using a rocking

motion and firm upward pressure (Caution: Be careful to avoid sharp ends of the blades). Lift the group out of the block and place it anterior-side up on the frozen glass plate. Place dry ice adjacent to blades to chill sections completely before separating. (**F**) Separate blades and lay sections out in order. (**G, H**) Sections can be removed from blades by flexing blade slightly with fingers in the direction of the arrows and pushing the section off with a second cold blade. (**I**) Some sections may require removal by slightly warming the razor blade between the fingers and thumbs and then quickly slicing off the frozen section with a second cold blade.

Figure 3: Determining ROIs using landmarks and the Allen Mouse Brain Atlas. In this example, frozen brain sections are on the left with corresponding plates from the Allan Mouse Brain Atlas on the right. ROIs (red dotted outlines) are identified by comparing visible landmarks (black shapes) to the Allen Mouse Brain Atlas. Landmarks include: fa and cc, corpus callosum, aco, anterior commissur, VL, lateral ventricle, V3, third venticle. Example ROIs: Pl/Il, prelimbic and infralimbic cortex, ACB, nucleus accumbens, MOs, motor cortex, CP, caudoputamen, HPF, hippocampus/dentate gyrus. Coronal atlas images on the right credit: Allen Institute. Images are from plates 40, 44, 55 and 71 (top to bottom) and were obtained from https://mouse.brain-map.org/experiment/thumbnails/100048576?image_type=atlas.

Figure 4: Dissecting ROIs. (**A**) Sections are handled using forceps that are chilled periodically on dry ice. Both sides of each section should be compared with the corresponding atlas images before dissection. (**B**) ROIs are removed from brain sections with cold scalpel or (**C**) biopsy punch.

Figure 5: Analysis of RNA and protein using frozen brain dissection method. (**A**) Example of poor versus good quality RNA. Electrophoretic separation of sample 1 displays degraded RNA with weak 28S and 18S bands and degradation products between 25 and 200 nucleotides (nt). Sample 2 shows good quality RNA with strong 28S and 18S bands and little signal at low molecular weights. RNA integrity numbers reflect this difference with sample 1, RIN = 2.5 and sample 2, RIN = 8.6. (**B**) Comparison of RNA extracted from frozen versus freshly dissected medial prefrontal cortex of mice. Frozen samples were kept at -80 °C for several months before dissection. Fresh samples were processed immediately following harvest. RIN values from samples collected using both methods are high, with strong 18S and 28S bands indicating that nucleic acid has been preserved. (**C, D**) mPFC was dissected from frozen brains and RNA or protein was extracted. (**C**) RNA analysis shows good quality RNA was obtained for all twelve samples with high RINs and little degradation product observed. (**D**) Western blot analysis was done on total protein and was probed for the presence of the high molecular weight KCC2 ion channel (red bands, MW = 130 kDa). The KCC2 band is clearly visible with no lower molecular weight breakdown products observed. The housekeeping gene, Actin (green bands, MW = 42 kDa) is also shown. mPFC samples from 12 brains are labeled 1-12. WB stands for whole brain control.

DISCUSSION:

This work describes a technique to isolate small, specific regions of brain while limiting degradation of nucleic acid and protein. Damage to brain tissues happens quickly once an organism dies. This is partially due to a rapid buildup of extracellular glutamate and the resultant excitotoxicity that occurs[21]. Messenger RNA is particularly vulnerable to degradation[22,23].

Breakdown of protein and nucleic acid is greatly reduced at low temperatures as evidenced by a recent article describing biological activity in mammoth cells frozen in permafrost 28,000 years ago[24].

With the method described herein, brains are snap frozen and tools and tissues are kept below freezing until nucleic acid or protein extraction is started. Frozen ROIs are then homogenized in buffer containing protease and nuclease inhibitors, protecting target molecules from degradation at higher temperatures.

In this process, it is important to identify clear landmarks to pinpoint ROIs within the sections. Fiber tracts of the anterior commissure and corpus callosum work well for this purpose, as do the ventricles. Changes in the shape of the hippocampus as one moves anterior to posterior can also be used. Depending on how well the brain is aligned in the matrix, landmarks may be skewed, or missing. Caution should be taken before collecting ROIs from sections like this as contaminating regions may end up in the sample. Before collection, an ROI should be checked on both sides of the section to make sure it is consistent throughout the section. Dissection of ROIs is limited to well defined areas of the brain that are greater than approximately 1 mm in the x-y dimension.

When dissecting ROIs, there is a need to keep tissues frozen without making them too brittle. Tissue that is too cold will fracture when pressure is applied with a punch or scalpel. The region of interest is often hard to identify from other fragments when this happens. One way to mitigate this is to briefly move the sections to a warmer area of the plate (away from dry ice). It is also important to keep the glass plate clean. Once a section is processed, the plate should be scraped clean with a razor blade before moving on to the next. Over time, water will condense onto the dissection plate from the air which may cover or otherwise interfere with sample recognition. Cleaning the plate is done by scraping the sharp edge of a razor blade across the surface of the glass and wiping up the collected frost and debris. Small ROIs become indistinguishable from unwanted brain fragments so it is very important to maintain a clean working area.

Many labs use commercial products[25] to protect RNA in harvested tissues. While labile molecules are well preserved this way, one drawback is that the morphology of the tissue is radically changed. As a result, it may be difficult to find the regions of interest using reference maps. Dissecting ROIs from snap-frozen brains before using these products may therefore be a better option.

Although we have focused on coronal slices in this work, this method should work well for horizonal and sagittal sections. Maps for these orientations are generally lower in resolution as compared to those for coronal sections. However, this is changing rapidly with initiatives from groups like the Allen Brain Institute. Another consideration is the age of the animal at harvest. The morphology of young animal brains is very different from that of the adult and will require a specialized atlas and a different size brain matrix. Resources like the Developing Mouse Brain Atlas[26] are essential in collecting accurate brain samples from younger mice.

This work describes a process that can be used to harvest small regions from within the rodent

brain. The morphology of sections is well preserved with frozen slicing, and as the tissues are kept frozen from harvest to extraction, the quality of nucleic acid and protein is high. This process has the advantage over the dissection of freshly collected tissue as sample brains from a large group of animals can be quickly collected and stored. Regions within brains can then be identified using landmarks identified in common brain atlases and collected at a careful pace without losing target molecules. This method may be a good option for some labs as the tools and regents needed are inexpensive and readily available.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

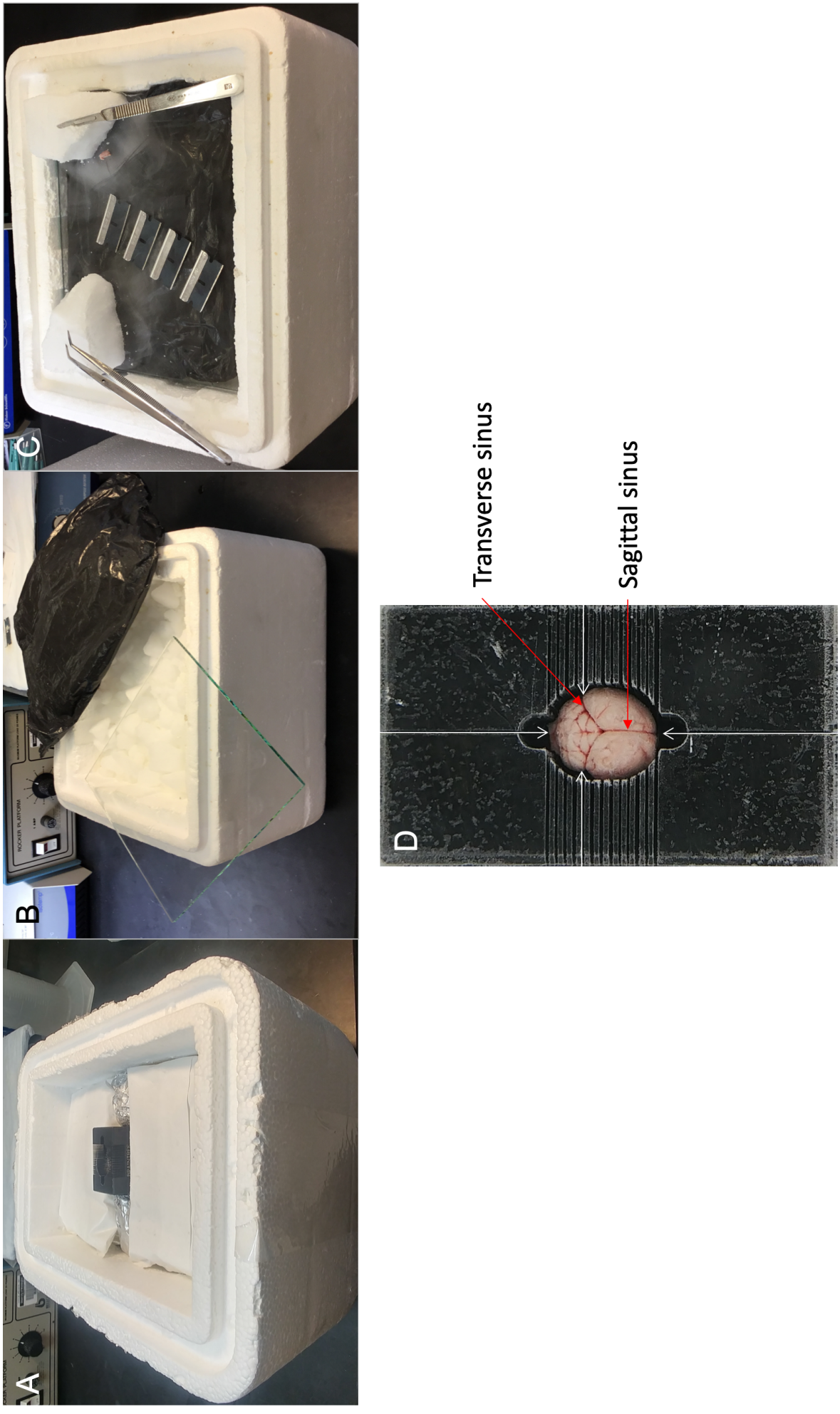

Figure 2

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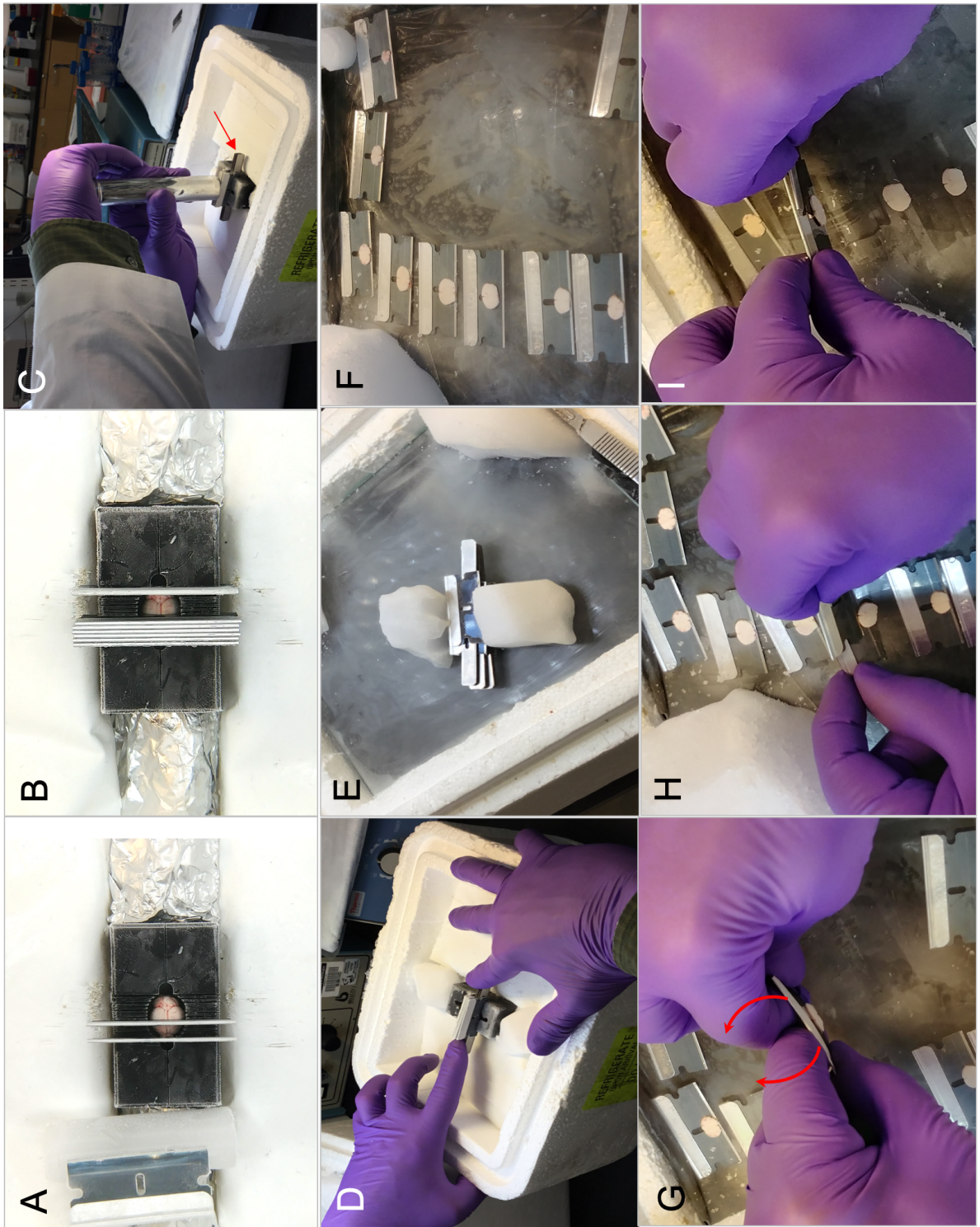

Figure 3

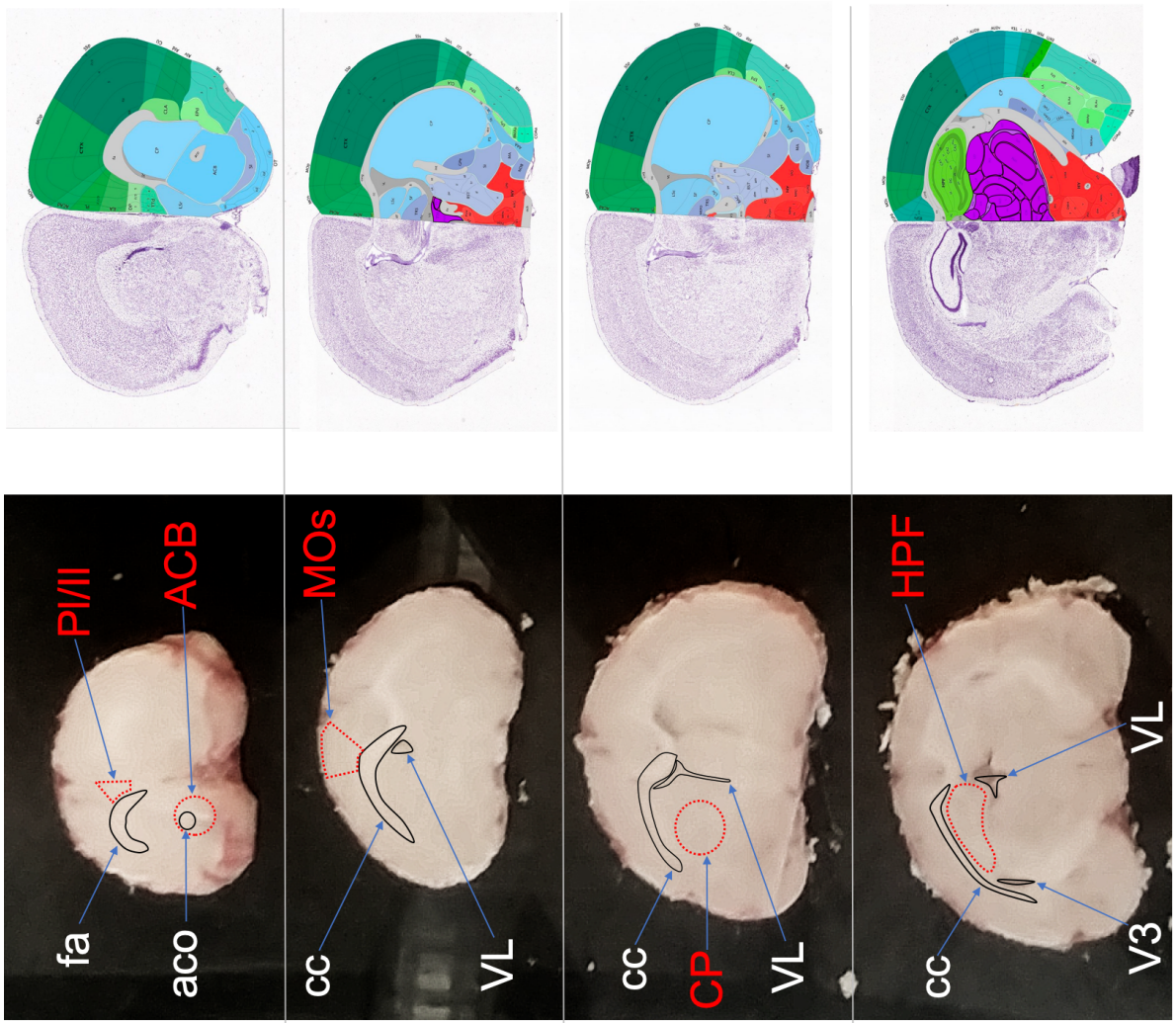

Figure 4

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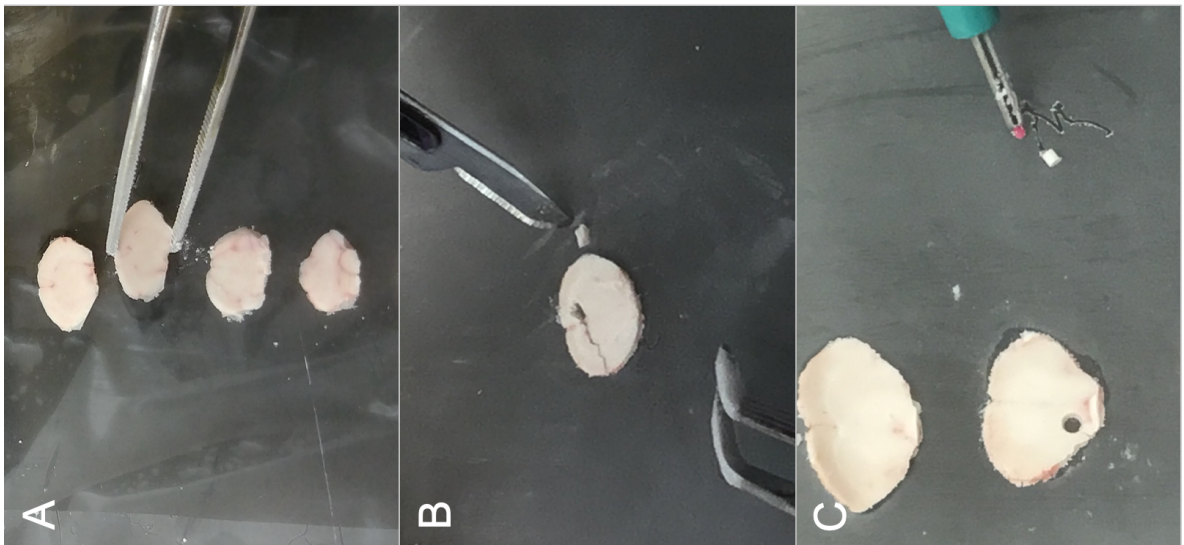

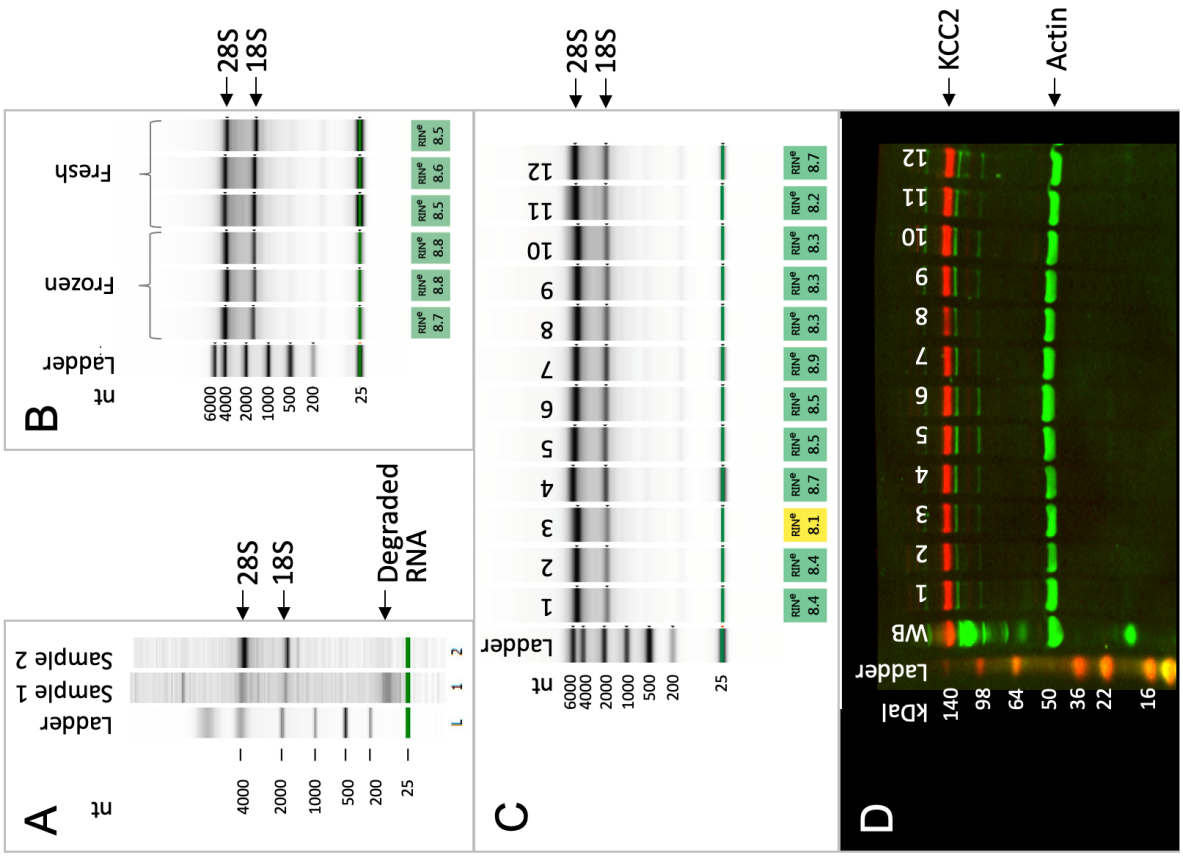

Name of Material/ Equipment	Company	Catalog Number
0.5 mm Mouse coronal brain ma	Braintree Scientific	BS-SS 505C
0.5 mm Rat coronal brain matric	Braintree Scientific	BS-SS 705C
1.0 mm Biopsy Punch with plung	Electron Microscopy Sciences	69031-01
1.5 mL microcentrifuge tubes	Dot Scientific	229443
1.5 mm Biopsy Punch with plung	Electron Microscopy Sciences	69031-02
2.0 mm Biopsy Punch with plung	Electron Microscopy Sciences	69031-03
4-12% NuPage gel	Invitrogen	NPO323BOX
Bioanalyzer System	Agilent	2100
Dounce tissue grinder	Millipore Sigma	D8938
Dry Ice		
Fiber-Lite	Dolan-Jenner Industries Inc.	Model 180
Glass plates	LabRepCo	11074010
HALT	ThermoFisher	78440
Low profile blades	Sakura Finetek USA Inc.	4689
mouse anti-actin antibody	Developmental Studies Hybridoma Bank	JLA20
Nanodrop	Thermo Scientific	2000C
No. 15 surgical blade	Surgical Design Inc	17467673
Odyssey Blocking buffer	LiCor Biosciences	927-40000
Omni Tissue Master 125	VWR	10046-866
rabbit anti-KCC2 antibody	Cell Signaling Technology	94725S
RNA Plus Micro Kit	Qiagen	73034
RNaseZap	Life Technologies	AM9780
Scalpel handle	Excelta Corp.	16050103
Standard razor blades	American Line	66-0362

TRIzol Reagent	ThermoFisher Scientific	15596026

Comments/Description

Cutting block

Cutting block

For storing frozen ROIs

protein gradient gel

RNA analysis system

Glass tissue homogenizer

Cool lamp

protease inhibitor cocktail

Antibody

Used in initial RNA purity analysis

Western blocking reagent
Tissue homogenizer
Antibody
Used to extract RNA from small tissue samples

Used to extract RNA from tissue

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Title of Article: A Procedure to collect frozen brain regions for downstream analyses

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Added text:

Process frozen tissue collected in this manner by adding cold RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% CHAPS and protein inhibitor cocktail (see **Table of Materials**)) for protein extraction, or a guanidinium-containing solvent (see **Table of Materials**) for RNA extraction to the frozen sample and immediately homogenize using either a glass dounce or mechanical homogenizer (see **Table of Materials**). In this way, protease and nuclease inhibitors are in place as the sample warms to protect protein and nucleic acids from degradation.

Commented [A1]: How exactly?

3.3. Adjust the brain's position in the matrix with cold forceps so that the sagittal sinus and transverse sinus line up with the perpendicular grooves of the block (**Figure 1D**). This will help ensure symmetrical sections for easier dissection. Touch tips of forceps to dry ice briefly to chill before adjusting brain.

(D) To ensure symmetric sections, the brain should be positioned with cold forceps so that the sagittal sinus and transverse sinus line up with the perpendicular grooves of the block (white arrows).

Figure 3: Determining ROIs using landmarks and the Allen Mouse Brain Atlas. In this example, frozen brain sections are on the left with corresponding plates from the Allan Mouse Brain Atlas on the right. ROIs (red dotted outlines) are identified by comparing visible landmarks (black shapes) to the Allen Mouse Brain Atlas. Landmarks include: fa and cc, corpus callosum, aco, anterior commissur, VL, lateral ventricle, V3, third venticle. Example ROIs: Pl/Il, prelimbic and infralimbic cortex, ACB, nucleus accumbens, MOs, motor cortex, CP, caudoputamen, HPF, hippocampus/dentate gyrus. Coronal atlas images on the right credit: Allen Institute. Images are from plates 40, 44, 55 and 71 (top to bottom) and were obtained from https://mouse.brain-map.org/experiment/thumbnails/100048576?image_type=atlas.

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