

Techniques for Anatomic Imaging of Mouse Brain:

Robia G. Pautler, Ph.D.

Dept. Molecular Physiology and Biophysics, Dept. Neuroscience, Dept. Radiology,
Translational Biology and Molecular Medicine Program,
Baylor College of Medicine
Houston, TX 77030

ABSTRACT:

The main goal for anatomical brain imaging is to obtain adequate contrast between regions of interest. Some general categories for brain imaging include distinctions between gray and white matter, diseased/injured vs. healthy regions, areas of activation, regions of inflammation, areas of protein accumulation/loss or white matter projections. Brain imaging in the mouse brain is of particular interest due to the multiple mouse models of human disease that are available. However, mouse brain imaging also brings with it challenges. Different strategies for anatomical mouse brain imaging are discussed here.

INTRODUCTION:

I. Maintaining a Mouse in the Magnet -- *In Vivo* vs. *In Vitro*

The first assessment that must be made in a mouse brain imaging experiment is whether the experiment should be conducted *in vivo* or *in vitro*. *In vivo* imaging clearly holds the advantage of the tissue being in the most pristine anatomical and physiological condition with the disadvantage of potential motion artifacts. During *in vivo* imaging, it is important to consider and maintain adequate respiratory and cardiac rates, temperature, hydration states and blood glucose levels (see **Table 1**). Furthermore, the eyes must be cared for and covered with an eye ointment prior to loading into the magnet; otherwise, the eyes will dry out (evidenced by a white spot on the eye) accompanied with the risk of severe damage to the eye.

Mice that are maintained in the magnet for greater than 4 hours should be hydrated via intraperitoneal lines or intravenous lines. Additionally, in the case of physiological studies, proper glucose levels must also be maintained for longer studies. General physiological characteristics of the laboratory mouse are listed in **Table 1**.

TABLE 1: Standard Physiological Parameters for Mouse

GENERAL ^{1,2,3}	
Body Weight	20-30 g; highly variable
Body Temperature	~36.9 ⁰ C
Blood Glucose Levels	between 60-130 mg/dl
Lifespan	1.3 – 3 years; highly variable
CARDIOVASCULAR ^{1,2,3}	
Total Serum Protein Content	~4-7g/100 ml
Blood Volume	~5.5 ml per 100 g body weight. A 20 g mouse will only have a blood volume of ~ 1.1 ml!
Heart Rate	Adult: 632±51.3 beats per minute (unanesthetized) Newborn: 286±56.8 beats per minute (unanesthetized)
Systolic Pressure	83-164 mm Hg
Blood pH	7.3 – 7.4
RESPIRATION ^{1,2,3}	
Respiratory Rate	~163 breaths per minute (unanesthetized)
Tidal Volume	~0.15 ml

An additional factor to consider for *in vivo* imaging is anesthesia choice. There are multiple choices of anesthesia ranging from inhalation anesthetics to injectables, analgesics and sedatives. It is important to note that after the introduction of anesthesia, the physiological values such as respiratory and heart rate may (likely) deviate from the listed values in **Table 1**.

Inhalation Anesthetics are the preferred choice of anesthesia for anatomical studies in that they are relatively safe and very effective. Anesthesia depth can easily be controlled. For functional

brain studies, the anesthesia impact on the neurons must be evaluated carefully when the study is being designed. Some common inhalation anesthetics are listed in **Table 2**.

TABLE 2:

COMMON INHALATION ANESTHESIAS <small>adapted from 4</small>				
Drug Name	MAC	Response	Toxicity	Notes
Isoflurane	1.5 (we usually maintain our mice on 2 – 2.5)	Fast	None	++ RD + CVD
Halothane	0.9	Moderate	Hepato- and nephrotoxicity in hypotensive animals	++ CPD and a risk of malignant hyperthermia in some strains

*MAC: Minimal Anesthesia Concentration is the % concentration of the drug required to anesthetize 50% of the animals. RD = Respiratory depression. CVD = Cardiovascular depression. CPD = Cardiopulmonary depression.

There are also multiple **injectable anesthetics** that can be administered IV, IM, IP or SC.

Some of the more common injectable anesthetics are listed in **Table 3**.

TABLE 3:

COMMON INJECTABLE ANESTHESIAS <small>adapted from 4</small>				
DRUG	TYPE	ROUTE	RESPONSE	NOTES
Pentobarbital	Barbituate	IV (50-75% dose) or IP (100% dose)	Anesthesia lasts 45 – 120 minutes. Prolonged recovery	Easy to overdose animal. Can be supplemented with analgesics. IP administration can irritate injection area.
Alpha Chlorolose/ Chloral hydrate	Mild hypnotic	IV (recommended) or IP (not recommended due to adynamic ileus)	Short acting (1-2 hours)	Minimal CPD. Can only be utilized for non-painful procedures. Not recommended if alternative drugs are available.
Urethane	Other	IP or IV	Long acting (8-10 hours)	Minimal CPD. Carcinogenic and only for terminal studies.
Avertin	Other	IP	Rapid induction and recovery (90 minutes for complete recovery)	Can be associated with abdominal adhesions resulting in high mortality. Use discouraged. Not available commercially.
Ketamine/Xylazine	Dissociative Anesthetic	IM, IP or IV	Short acting (30 minutes to 1 hour)	Good for restraint and painful procedures. Some CPD and RD. Can re-dose.

For *in vitro* imaging, it is important to note that brain tissue deteriorates quickly and the mouse should be immediately euthanized prior to imaging if no fixative is introduced. The mouse should be kept chilled during imaging (do not turn on the heating system) to preserve the integrity of the tissue. However, even better would be the use of fixed tissue (*A perfusion protocol and ketamine/xylazine preparation protocol by Ms. Taneasha Washington in Dr. Pautler's lab is attached as a supplement at the end of this document*). Typically, it is good practice to maintain the brain in the skull to preserve the structural integrity of the brain tissue. However, there are additional important considerations to take into account that are detailed in the methodologies section.

Once the decision of *in vivo* or *in vitro* imaging has been made, the types of information desired and hence, the type of imaging modality must be decided upon. Some of these approaches are summarized in the following methodologies section.

METHODOLOGIES:

II. Obtaining Optimal Contrast in the Brain: Making the Most of Endogenous Contrast.

Perhaps the best exercise for any new student interested in rodent brain MRI imaging is to read through and reproduce the work of Ting and Bendel⁵. In this paper, they calculated the imaging parameters that would yield optimal contrast to noise (C/N) between gray matter, white matter and cerebral spinal fluid (CSF) in spin echo images at 4.7T. The C/N between *tissue a* and *tissue b* is defined as:

$$(C/N)_{ab} = P(S_a - S_b)/\sqrt{TR}$$

where P is a proportionality constant. For example, the signal intensity of *tissue a* is designated as S_a and defined as:

$$S_a = WD_a E_{1a} E_{2a}$$

where

$$E_{1a} = 1 - \exp(-TR/T1_a)$$

and

$$E_{2a} = \exp(-TE/T2_a)$$

The division by the square root of the TR normalizes the expression for a constant imaging time and WD_a refers to the water density in *tissue a*. The results of such an exercise are useful for any laboratory working at any magnetic field strength as optimal imaging parameters for normal anatomical brain imaging can be acquired [Figure 1]. A similar approach can be employed to any regions of interest (e.g. healthy vs. disease) to optimize the endogenous contrast. It should also be noted comparable approaches can be applied to different imaging sequences as well. Additionally, the gain in C/N should be weighed against the total imaging time.

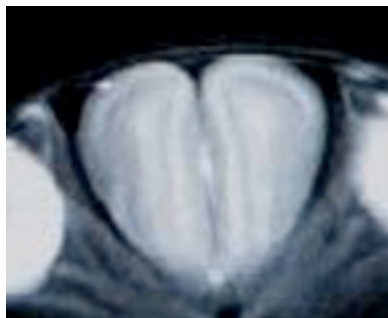


Figure 1: This is an example of mouse olfactory bulb images (without contrast) that were acquired on a 7.0T Bruker Avance Biospec with a 1 cm surface coil at Carnegie Mellon University by Dr. Pautler. The T_1 s and T_2 s of the different layers were measured and optimal C/N was calculated as detailed by Ting and Bendel⁵. Note the differentiation between the different layers within the olfactory bulbs. Spin Echo Imaging sequence, TR = 1250ms, TE = 23 ms. NA = 2. matrix = 256x256. slice thickness = 1 mm.

III. EXOGENOUS CONTRAST: “MRI HISTOLOGY” AND MEMRI TRACT TRACING

Oftentimes, endogenous contrast is not enough to highlight the areas of interest in the mouse brain. This necessitates the introduction of exogenous contrast agents. Three common strategies are briefly detailed here:

A. “Staining” with Chelated Gd^{3+} : Drs. Allan Johnson and J.M. Tyszka both have utilized chelated Gd^{3+} to act as an MRI histology agent^{6,7}. The methods by Dr. Johnson included perfusing the animal simultaneously with fixative and gadoteridol resulting in outstanding contrast between gray and white matter⁶. Dr. Tyszka, on the other hand, perfusion fixed animals, removed the head and rocked the sample overnight at 4⁰ C overnight⁷. Subsequently, the sample was transferred to a 5mM gadoteridol and 0.01% azide solution and rocked at 4⁰ C for approximately 2 weeks. Prior to imaging, the samples were brought up to room temperature. Dr. Tyszka observed that this soaking in gadoteridol significantly reduced the T_1 of the sample and reduced the total imaging time for a given resolution and targeted signal to noise ratio. Dr.

Tyszka applied this method towards assessing the shiverer mouse and in comparison to controls, observed differences in the tensor trace and both axial and radial diffusivity⁷.

B. Manganese Enhanced MRI (MEMRI) Tract Tracing: A very important feature about the manganese ion (Mn^{2+}) is that it is paramagnetic⁸⁻¹². The paramagnetic properties of Mn^{2+} effectively shorten the spin-lattice relaxation time constant, T_1 , in tissues where Mn^{2+} has accumulated⁸⁻¹². This results in positive contrast enhancement in T_1 -weighted MRI images where Mn^{2+} has accumulated⁸⁻¹². The following combination of three features of Mn^{2+} prompted the initiation of a small MRI pilot study in the olfactory system of the mouse to determine whether the Mn^{2+} ion could be used to trace neuronal tracts in an activity-dependent manner:

1. Mn^{2+} can be used to measure Ca^{2+} influx.
2. Mn^{2+} can be transported along axons and traverse synapses.
3. Mn^{2+} is paramagnetic and, therefore, MRI detectable.

The initial MEMRI tract tracing study involved exposing mice to a nasal lavage of a concentrated Mn^{2+} solution and imaging at time points after exposure¹³. The olfactory pathway in the mouse exhibited contrast enhancement in T_1 -weighted MRI images, similar to what Tjälve et al observed in pike and later rat¹³⁻¹⁵. The peak signal intensity in the olfactory bulbs occurred at 29 h after Mn^{2+} exposure, and after 72 to 96 h, the signal intensity returned to baseline¹³. The return to baseline signal intensity, indicating the wash-out of the ion from the tissue, opens up the possibility for longitudinal studies to be performed in the same animal¹³. Additionally, it is important to note that the levels of Mn^{2+} used in these studies were all well below the neurotoxic levels and, indeed, no symptoms of manganism were ever observed in any of the test subjects. The olfactory lavage protocol is as follows: **1.** Dissolve 0.77 g of MnCl_2 into 1 mL of sterile phosphate free buffer. Vortex. The solution will be a faint pink; **2.** Anesthetize a mouse and, on lack of a toe pinch reflex, pipet 1 μL of the MnCl_2 solution into the naris using a 10- μL pipetter. Pipet 1 μL into the contralateral naris. Repeat until up to 2 μL per naris have been applied; **3.** Recover the mouse on a warming pad until the mouse awakens. Image the mouse either 1 hour later for dynamic MEMRI transport studies or 24 hours later for anatomical MEMRI tract tracings.

C. Manganese Histology: Drs. Afonso C. Silva and Alan Koretsky have been instrumental in developing the use of MEMRI for “MRI histology”¹⁶. For example, they have elegantly demonstrated the detection of rodent cortical laminar architecture as well as other deep brain

nuclei utilizing MEMRI. The MEMRI histology methods for mouse are as follows¹⁶: 1) IV administration of 175mg/kg (884.3 μ mol/kg) isotonic solution of 120mM MnCl₂•4H₂O utilizing a 30 gauge butterfly needle at a rate of 250 μ l/h in an anesthetized mouse. 2) Recover mouse for 24 hours and image utilizing a T₁-weighted sequence.

IV. METHODS TO INTRODUCE EXOGENOUS CONTRAST AGENTS INTO THE BRAIN.

Oftentimes, contrast agents do not have direct access to the brain due to the presence of the blood brain barrier (BBB). There are two main ways to introduce contrast agents into the brain. These include transiently breaking the BBB with mannitol or by directly injecting the agents into the brain. Both of these methodologies are detailed here.

A. MANNITOL INFUSION: Mannitol is used to transiently open the BBB. Mannitol acts by temporarily shrinking the tightly coupled endothelial cells that comprise the BBB. This is referred to as “breaking the BBB”. This is achieved by infusing in a hyperosmotic concentration of mannitol. The parameters as detailed in the outstanding work of Dr. Ichio Aoki and Dr. Alan Koretsky are¹⁷: a bolus injection of 25% D-mannitol solution (5 ml/kg) via the right carotid artery¹⁷. Dr. Aoki’s protocol includes infusing mannitol after the first set of control images and 10.6 minutes after MnCl₂ infusion for dynamic Activity induced manganese-dependent contrast (AIM) studies¹⁷.

B. STEREOTAXIC INJECTIONS (adapted from 18).

It is also possible to introduce contrast agents or labeled cells directly into the brain parenchyma. The methods for stereotaxic injections into mouse brain are as follows: **1.** Anesthetize the mouse with 7.5 mg/mL of ketamine and 0.5 mg/mL of xylazine at a dose of 0.17 mL/10 g of body weight. Alternatively, 5 mg/mL of sodium pentobarbital can be used as an anesthesia at a dose of 0.1 mL/10 g of body weight. **2.** On lack of a toe pinch reflex, put the mouse on a paper towel to trim the fur from the top of the head. **NOTE:** *It is not trivial to remove the fur from the mouse’s head before surgery. Mice are different from rats in that the use of an electric shaver to remove the hair does not work well (even electric moustache clippers have been tried with dismal results!). Two alternatives are available—either use scissors to trim away the excess fur or some researchers use a scalpel blade to scrape away the fur. Because the scalpel*

blade can oftentimes lead to the simultaneous removal of skin, it is strongly recommended to use scissors to trim the hair. Use the scissors to lift up the hair at the base of the roots such that the scissors are perpendicular to the plane of the head and trim as closely as possible to the scalp. Continue removing the hair on the entire top of the head, from the base of the skull to just behind the eyes, extending laterally to the ears.

3. Swing the microscope secured on the boom out of the way (see **Figure 2** for the basic setup).
4. Place the mouse into the stereotaxic device, and secure the nose, tooth, and ear bars. At this point, put an eye ointment solution onto the eyes, otherwise, they can dry out during the surgical procedure. To ensure the sterility of the surgical procedure, it is advisable to put sterile surgical drapes over the body of the mouse, leaving only the shaved head exposed. At this point, use surgical scrub and sterile cotton swabs to clean the head. After cleaning the scalp area, use a surgical rinse or sterile H₂O to remove the soap. To ensure sterility, this cleaning must be performed a total of three times.
5. Make sure that the head is level. Once the initial incision has been made and the skull has been dried of blood and fluids, the longitudinal and the lateral levelness of the head can be validated. Use a magnifying visor rather than the microscope during this procedure.
6. Secure a pulled pipet to the stereotaxic apparatus and use it to map out the stereotaxic coordinates relative to bregma. Again, use the magnifying visor during this procedure. While wearing the magnifying visor, it is also helpful to use a small, handheld dental mirror that can easily be placed at different locations to view the pipet from different angles. Once the site has been identified, use a fine, highspeed drill to drill a hole just through the skull, taking care not to touch the brain tissue with the drill bit.
7. Lower the filled needle into the correct coordinates in the brain. Set the injection pressure to approx 20 psi and set the injection time to about 5 ms.
8. Swing the microscope back toward the stereotaxic apparatus to watch the meniscus during the injection. Adjust the location and focus of the scope until the gage can be observed through the microscope. Zoom in as much as possible to the meniscus and use a gage attached to the back of the needle to monitor the injection volume (**Figure 2**). With an initial injection time of 5 ms, adjust the injection timing (usually increase it) such that the meniscus only moves at the most 1/10 of a line width, as observed on the calibrated gage. Inject until the desired volume is expelled. The optimal injection volume for tract tracings in the mouse brain appears to be 10 to 20 nL. Leave the needle in place for approx 5 min.
9. Before closing the wound, place a few drops of an analgesic (such as bupivacaine) underneath the scalp (typically on the side of the skull contralateral to where the

hole was drilled). Be sure not to get this drug on the exposed brain tissue. **10.** Use sterile wound clips or suture to close the wound. If wound clips are used, be sure to remove them before imaging. **11.** Place the mouse on a warming pad as it recovers from the anesthesia and surgery. Once the mouse has awakened, it can be returned to its cage until it is to be imaged. An example of a deep brain tract tracing is demonstrated in **Figure 3**¹⁹.

Figure 2:

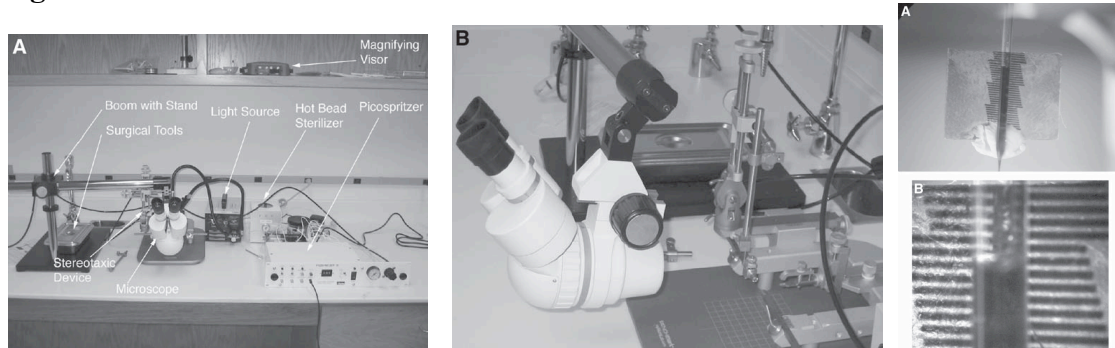


Figure 3:

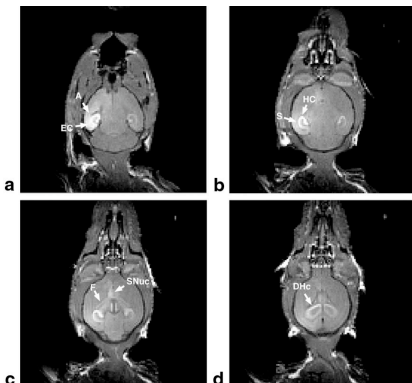


Figure 2: (left) An example of a stereotaxic setup. (Middle) The middle image includes a demonstration of how to position the dissecting microscope on a boom to observe the injection needle and meniscus in the pipet. (Right) View of the pipet with a gage attached as well as a zoomed in view with the scope demonstrating a view of the meniscus against the gage. This allows for close monitoring of injection volumes. Figure adapted from reference 18.

Figure 3: Example of a MEMRI tract tracing resulting from a 20 nl injection of $MnCl_2$ into the amygdala. Figure adapted from reference 19.

V. Concluding Comments: Image Data Validation

Designing mouse brain imaging experiments is a multi-tiered process ranging from maintaining a mouse in the magnet to appropriate anesthesia choices to proper surgical methods to choosing an appropriate imaging sequence. Once the gauntlet has been successfully run and images are acquired and processed, a careful interpretation of the data ensues. This is near impossible without the addition of non-imaging validation of the data such as histology, behavioral assays and molecular or biochemical assays. It is imperative that we as imaging scientists make the effort to move beyond being regarded as mere image generators.

A few common histological, behavioral and molecular/biochemical assays that can be utilized to validate imaging data are listed in **Tables 4 – 6**. Note that the listed examples are just the tip of

the iceberg. An in depth description of each of these assays is beyond the scope of this document. References 20-23 are recommended references on these topics.

TABLE 4:

A FEW COMMON HISTOLOGICAL STAINS:		
STAIN	INFORMATION	OUTPUT
Hematoxylin and Eosin (H&E)	Hematoxylin stains basophilic structures (nucleic acid containing structures) Eosin stains eosinophilic structures (containing intracellular or extracellular proteins).	Hematoxylin: Purple (e.g. ribosomes and nucleus) Eosin: Pink to red. (e.g. cytoplasm. Red blood cells stain bright red)
DAPI	Used to stain DNA	Bright blue
Cresyl Violet/Luxol Fast Blue	CV: Aniline dye that preferentially stains RNA and DNA LFB: Lipids, myelin	Luxol fast blue, has an affinity for lipids,. When coupled with cresyl violet, neuron myelin stains blue, red blood cells turquoise, and the nuclei and Nissl bodies (primarily rough endoplasmic reticulum) stain as purple.
Prussian Blue	Stains for Iron	Beautiful blue color
Periodic Acid Schiff (PAS) Staining	Stains nuclei, acid mucosubstances, polysaccharides, gangliosides and muopolysaccharides.	Nuclei stain blue, Acid mucosubstances brightly, light-blue; Polysaccharides, neutral mucopolysaccharides are purple ⁶

TABLE 5:

A FEW COMMON MOLECULAR AND BIOCHEMICAL ASSAYS:		
ASSAY NAME	INFORMATION	OUTPUT
Western Blots	Detects the presence or absence of proteins in a tissue extract. Run on an SDS gel. Requires the use of a specific antibody against the protein of interest. Can also detect phosphorylation state of protein.	Band will be present or absent in the gel. Band intensity may also vary depending upon protein concentration.
Enzyme-Linked Immunosorbent (ELISA) Assay	Detect the presence of an antibody or an antigen in a sample. Performed in multi-welled plates coated with “capture antibody”. Sample added. If protein of interest is present, then will bind to antibody.	Add detection antibody for readout. Use biotin/avidin or fluorescence for protein/antibody detection. Readout in microplate reader.
Quantitative RT-PCR	Way to quantify mRNA in a sample. Need correct primers for PCR amplification.	Products are run on a gel. Bands are quantified utilizing a phosphoimager for example and subtracted from background and compared to controls.

TABLE 6:

A FEW COMMON BEHAVIORAL ASSAYS		
ASSAY NAME	INFORMATION	OUTPUT
Rotarod	Assesses motor coordination and balance, as well as motor learning	Amount of time mouse spends on rotating rod as well as length of time mouse learns to stay on the rod.
Open Field Locomotion	Motor function assessment.	Spontaneous activity of a mouse in an open field. Horizontal and vertical movements are recorded
Morris Water Maze (MWM)	Spatial learning and memory test. Two types: visible and hidden platform MWM. Hippocampal based.	Amount of time mouse spends in quadrant as well as number of times mouse crosses platform location, Record swim speed to ensure not a variable in data.
Fear Conditioning ()	Learning and Memory test. Amygdalar and hippocampal based. Two types: Contextual and Conditioned Fear.	Mouse freezes (stops moving) when it exhibits fear (indicating it has learned that either the context or condition will elicit a shock). Amount of time mouse exhibits freezing behavior is recorded.
Light Dark Exploration	Assesses anxiety-like behavior in rodents	Mice are tested in a box that contains a dark areas encompassing one-third of the box and a light area consisting of two-thirds of the box. The number of transitions between dark and light and the time spend in the light area are utilized as a measure for anxiety

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SUPPLEMENTAL INFORMATION:

Preparing Paraformaldehyde (PFA) for Transcardial Perfusions: 500mls Total

Ms. Taneasha Washington, Pautler Laboratory
Dept. Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030

*******Note when preparing any type of chemical please read your MSDS Book; to determine what types of safety clothing and equipment you should wear*******

1. Heat up 400mls of NanoPure/Mill Q water (**NAME OF WATER PURIFICATION SYSTEM**).
2. Gradually add 20grams of PFA (Sigma Cat: P6148) and stir to mix. *****Note the PFA should be stored at 4° for optimal usage. AND ALWAYS WEAR A MASK WHEN WEIGHING OUT PFA*******
3. Add 200ul 5N NaOH (The solution should become clear quickly; if not it means that the PFA is old)
4. Let the solution cool to room temp. (**I put the solution in an ice bucket for about 30min in order to cool down**).
5. When it's cool add 50mls of 10X PBS (Phosphate- Buffered Saline) (Sigma Cat: P3813)
6. Bring to final volume with NanoPure/Mill Q water. Stir to mix completely.
7. Adjust the pH of the solution to 7.2-7.4
8. I filtered the solution, using a bottle top filter system.

*******Note: Some Protocols suggested that you add sucrose to your PFA Solution; ****Add 5 % (25grams) Sucrose ***** you would add this after step # 5 if needed*******

30% Sucrose/PBS:

1. percentage: gram/per 100mls
2. Weigh out 30 grams of Sucrose (Sigma Cat: S5016)
3. Add sucrose to 100mls of 1X PBS. Stir to dissolve.

PBS (Phosphate- Buffered Saline) (Sigma Cat: P3813)

1. Empty one packet of buffer into 1liter of NanoPure/Milli Q water; Stir to dissolve(1 pk makes up a 1X solution)
2. Add a pinch Heparin(It's a very small amount so I don't normally measure it out I usually add 2 pinches to make sure the heparin is completely dispersed throughout the solution. Shake to mix. Heparin (Sigma Cat: H3393)

******Adding Heparin helps the blood not to clot during perfusion******

Make sure that when you perfuse your PBS + Hep Solution & PFA are ice cold (in an ice bucket covered with ice). We have noticed that you get a better perfusion this way ☺

Preparation of Ketamine/Xylazine anesthetic for the mice:

- .4mls of Ketamine (Controlled substance)
- .2mls of Xylazine
- 3.4mls of Physiological Saline Solution
- Total volume is 4mls

*******Notes Anesthesia*******

**Mixture= 0.75mg/ml ketamine +0.5mg/ml Xylazine
(Stock of ketamine=10mg/ml)/ (Stock of Xylazine 20mg/ml)**

- **So we have to use 0.3ml of ketamine for 4ml total**
- **And we have to use 0.1ml of Xylazine for a 4ml total**
- *******In reality we use 0.4ml of Ketamine +0.2ml of Xylazine to spike the anesthesia up a little bit, dilute in 3.4ml of Physiological Saline Solution for a total of 4ml) Then inject 0.1ml/per 10gram of body weight. Since a typical mouse is 30g we inject 0.3ml of anesthesia. *******

Protocol for Mouse Perfusion:

1. Prepare your solutions the night before and place them in the 4° degree but make up the anesthetic fresh for the day of usage.
2. Take out all of your solutions (1x PBS w/Heparin & 4% PFA+Sucrose Solution and remember to put them on ice (make sure that they are covered in ice to get a better perfusion.
3. Injecting the mouse with anesthetic:
Select the smallest gauge needle possible to limit tissue trauma and injection discomfort. A 25-30 gauge needle (we **used a 27 ½ gauge needle**) is recommended for use in a mouse. Before injecting, check for correct placement by pulling back on the plunger of the syringe to create a vacuum. This is known as aspiration.
Since a typical mouse is 30g we inject 0.3ml of anesthesia. **Refer to the notes about Anesthesia listed above.**
4. After injecting the mouse with the anesthetic wait about 5-10 minutes for the mouse to go down, check before cutting the mouse open by pinching it's toe or tail; if it still moves give the mouse a little more time; if it's longer than 15 minutes you can administer more anesthetic in small volumes (about .1 -.3cc) being careful not to kill the mouse.
5. A).First, secure the animal down with its belly facing up.
B).With your forceps, grab hold of the skin anteriorly to the urethral opening. Using your scissors, cut along the ventral midline from the stomach/mid area to the chest, being careful to only cut the skin and not the muscle wall underneath.

C). Next, make an incision starting from the rib cage area and cut along the rib cage on both sides of the animal. Also cut along the sternum. Basically, you will end up with an incision that looks very similar to an upside down Y.

D) Peel open the rib cage and hold it down with hemostatic clamps.

6. Now you are ready to perfuse, Using the peristaltic pump (Model # RP-1 FROM rainin: which is already set up for use with needle), insert the needle into the left ventricle of the heart and press the flow for the PBS + Heparin solution to flow through the blood stream (this is done in order to clear the blood out of the tissue) Once PBS/Heparin Solution flows through the blood circulation, The right atrium is going to swell; pinch it with scissors to make a hole in it and this will give you an out flow to the bloodstream. Keep the PBS/Heparin Solution flowing until the outflow is clear of blood and internal organs (heart & liver) become pale in color.
7. Now change the solutions to the 4% PFA+Sucrose Solution; let this flow to fix the tissue. ******NOTE**** It usually takes about 5 minutes in which the mouse limbs may be moving & twitching as they are being fixed**

8. Now you are ready to dissect the brain.

9. After the brain is out rinse 2x in 1x PBS (to clean off all the excess blood and debris. Place the brain in a vial with 4% PFA Solution and place in the 4° over night.

*******NOTE*****: Some Protocols suggested that you add sucrose; ****Add 5 % (25grams) Sucrose ***** you would add this after step # 5 if needed*******

10. The next day rinse the brain 4-5 times with 1XPBS to get rid of the PFA. After rinsing place the brain in the 30% Sucrose/PBS Solution overnight. ****** Keep in this solution until ready to cut*******

*******Remember to clean up the surgery area and tools and place the dead carcass in the -20*******