

Perfusion-Fixation of Adult Rat Brain

K. Harris Lab, Center for Learning and Memory, The University of Texas at Austin
Last updated on 4/13/2009 by M. Kuwajima.

At the end of this protocol is a Step-By-Step description of this procedure which may be sufficiently detailed for daily use after the excruciating detail in the next several pages have been read.

1. Reagents

- 50% Glutaraldehyde (Ladd Research 20211; 100 ml bottles; stored at 4°C)
- 20% Formaldehyde (Ladd Research 20304; 100 ml bottles; stored at RT)
- Sodium cacodylate trihydrate (Ladd Research 20305; stored at RT)
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma M5921; stored at RT)
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Sigma 223506; stored at RT)
- NaCl (Sigma S7653; stored at RT)
- KCl (Sigma P9333; stored at RT)
- NaHCO_3 (Sigma S6297; stored at RT)
- Na_2CO_3 (Sigma 223530; stored at RT)
- D-Glucose (Sigma G7528; stored at RT)
- Halothane (Sigma B4388; stored at RT)
- HCl and NaOH solutions (aq) to adjust pH.
- Additional reagents, e.g., buffers in which the brains are shipped, may also be required.

1.1. Prefix perfusate

Reagent	[final] mM	F.W.	For 2 L
ddH ₂ O	-	-	~1600 ml
NaCl	118.0	58.44	13.79 g
KCl	4.7	74.55	0.70 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2.0	147.02	0.59 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	4.0	246.48	1.97 g
NaHCO_3	12.5	84.01	2.10 g
D-glucose	11.0	180.16	3.96 g
Na_2CO_3^*	12.5	106.00	2.65 g

Oxygenated "Krebs-Ringer Carbicarb (KRC)" buffer is used to flush blood cells prior to fixative perfusion (Also to remove fixative from the tissue to improve immunoreactivity; optional). It is prepared from dry reagents as described left on the day before the procedure and stored at RT overnight.

*Dissolve the Na_2CO_3 in approximately 100 ml of water separately from the other reagents; DO NOT ADD it as a dry reagent directly to avoid solubility problems. When the other reagents have dissolved, slowly (approx. 2 ml at a time) add the

dissolved Na_2CO_3 with mixing. As Na_2CO_3 is added, it will be necessary to adjust the pH down to ~8 (preferably between 7.5 and 8) to prevent precipitation. After all of the Na_2CO_3 has been added, allow the solution to mix for several minutes before adjusting the buffer to pH 7.35-7.40, then bring the final volume to 2 L. Filter through a #5 Whatman filter paper into a 2-L bottle. Measure the osmolarity, and if necessary adjust it to 300-330 mmol/kg. The buffer is warmed to 41°C in a water bath and gassed with O₂/CO₂ (95%/5%) for 30 min before use.

1.2. Fixative

Reagent	[final]	F.W./Stock	For 2 L
ddH ₂ O	-	-	~1300 ml
Na cacodylate	100 mM	214.0	42.8 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2 mM	147.02	0.59 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	4 mM	246.48	1.97 g
formaldehyde	2.0%	20.0%	200 ml
glutaraldehyde	2.5%	50.0%	100 ml

Remove 50% glutaraldehyde from refrigerator (and leave at RT) 3 days prior to fixative preparation. The stock buffer minus the aldehydes is usually prepared the day before use, stored at RT, and the aldehydes are mixed in before warming to 41°C. Our standard perfusion fixative, 2% formaldehyde + 2.5% glutaraldehyde, is shown left.

After the salts are dissolved, adjust to pH 7.35, and store at RT. Move the solution to a fume hood before adding the aldehydes and bring the final volume to 2 L, filter through a #5 Whatman filter paper into a 2-L bottle. Warm the solution to 41°C in water bath before use.

2. Equipment/Supplies

Personnel Protective Equipment:

- Eye protection, face shield
- Gloves (nitrile)
- Lab coats

Air Pressure System:

- Air pump: Perfusion Two (MyNeuroLab.com)
- 1-L Kimax bottle × 1
- #6 two-hole stopper × 1
- Sphygmomanometer with a hand pump (cut off the cuff)
- Assorted tubing, connectors, and clamps

Oxygenation System:

- O₂/CO₂ (95%/5%) cylinder with regulator and support
- Assorted tubing, connectors, and clamps

Anesthesia System:

- Large glass dessicator, wad of 4-5 Kimwipes, Pasteur pipet
- Matrix VIP3000 Halothane Vaporizer (Stoelting)
- Harvard Apparatus Model 683 small animal ventilator
- Nose cone (Kent Scientific)
- 16-gauge endotracheal tube with Y-connector
- Assorted tubing, connectors, and clamps
- Optional: 10"×4"×4" Plexiglas anesthesia box (Stoelting)

Perfusate System:

- Deep water bath (e.g., VWR Digital Unstirred Water Bath, L×W×D = 12⁷/₈"×11¹³/₁₆"×5²⁹/₃₂"; VWR 89032-216)
- Lead donuts × 2
- 2-L Kimax bottles × 2
- #6 three-hole stopper × 2
- 3-way valve × 1
- Flow regulator
- 13-gauge needle with 60° bevel with cork disk ½ inch from tip (to restrict depth of penetration)
- Air stone × 2
- Assorted tubing, connectors, and clamps

Waste Collection System:

- 4-L vacuum flask
- #11½ one-hole stopper × 1
- Vacuum line filter (Whatman VACU-GUARD, VWR 28137-858)
- Assorted tubing and connectors

Dissection/surgical instruments (Figs. 1 and 8):

- Stainless steel dissection tray with Styrofoam board cut to fit snugly lengthwise in the tray with ~1-inch space on both sides.
- Assorted dissecting scissors
- Silk suture (or cotton tread from fabric store can be used instead)
- Forceps
- Hemostats
- Scalpel and blades
- Knife (to decapitate rat)
- Bone Rongers
- Spatulas



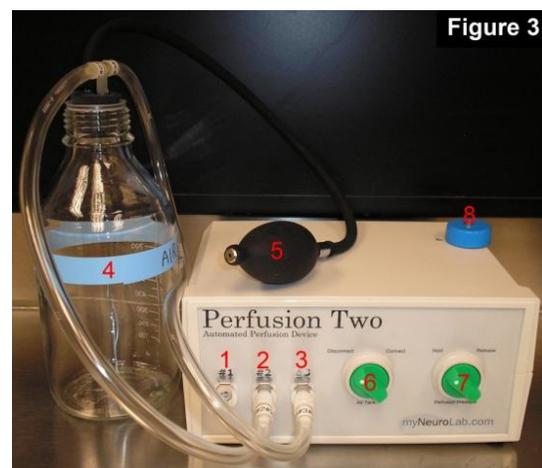
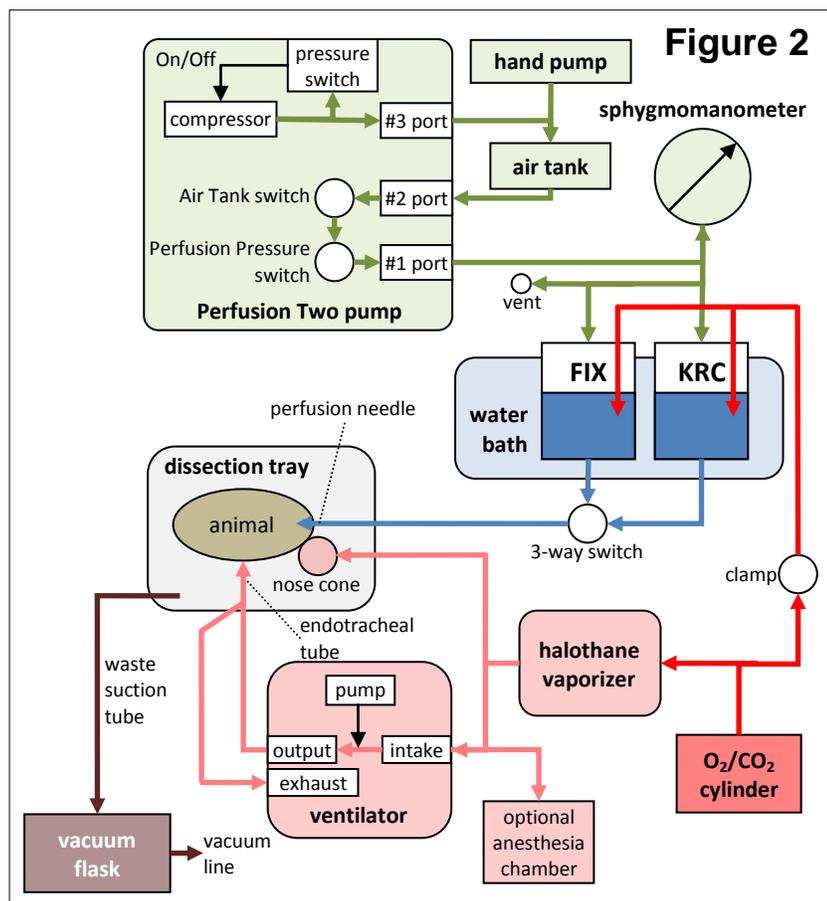
- Applicator sticks
- 3M Transpore surgical tape or needles to pin animal to Styrofoam board

3. Perfusion Apparatus Assembly

The perfusion apparatus consists of five sub-systems (Fig. 2): 1) air pressure (**Green**), 2) perfusate (**Blue**), 3) oxygenation (**Red**), 4) anesthesia (**Pink**), and 5) waste collection (**Brown**).

The air pressure system (Figs. 2 and 3) consists of a modified Perfusion Two air pump, air tank, and a modified sphygmomanometer. The air tank (1-L Kimax bottle; 4 in Fig. 3) is sealed with a #6 two-hole stopper. A Y- (or T-) connector is attached to one of the two holes in the stopper, to which two pieces of tubing are attached to connect the air tank to #2 and #3 ports of the Perfusion Two air pump (2 and 3 in Fig. 3, respectively). A hand pump (the bulb detached from the cuff of a sphygmomanometer; 5 in Fig. 3) is attached to the other hole in the stopper. #1 port of the air pump (1 in Fig. 3) is connected to the sphygmomanometer (7 in Fig. 4) and perfusate bottles (see below).

The perfusate system (Figs. 2, 4, and 5) consists of a water bath, two 2-L bottles (one for KRC and the other for the fixative), perfusion needle, assortment of tubing, connectors, clamps, and valves. Lead donuts (6 in Fig. 4) are placed around the necks of the bottles to keep them from floating and tipping over in the water bath as the fluid in them is exhausted. The fixative and the KRC bottles are sealed with #6 three-hole stoppers (Figs. 5 [for fixative bottle] and 6 [for KRC bottle]). The three holes are used to connect the bottles with 1) air pressure system, 2) oxygenation system, and 3) a 3-way switch and IV tubing leading to the perfusion needle. KRC and fixative are driven during perfusion by the air pressure system (see above). It is very important that the stoppers and the inside of the neck of the bottles be completely dry before inserting the stoppers to prevent the stoppers from blowing out under pressure. When the apparatus is assembled prior to each perfusion, it should be tested for the pressure of up to 200 mmHg to ensure the connections throughout the system will withstand pressure and not blow apart. Superglue is used to secure joints between different sized tubing.



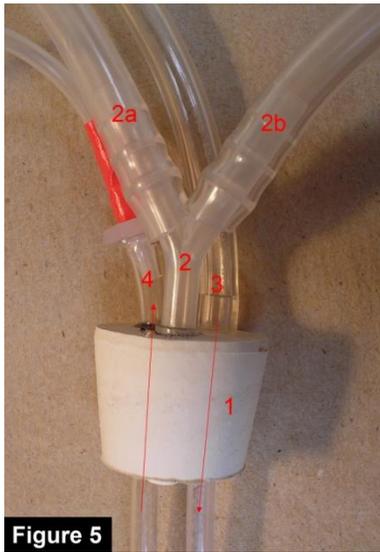


Figure 5

Connecting the perfusate bottles to the air pressure system: Attach the long end of Y-connector into one of the three holes in the stoppers (2 in Figs. 5 and 6). Use a short piece of Tygon tubing on one side of Y-connector (2b in Figs. 5 and 6) to connect the two perfusate bottles. On one of the stoppers, another piece of tubing (2a in Fig. 5) is attached to the unused side of the Y-connector to connect the sphygmomanometer (indicator; 7 in Fig. 4) and the #1 port of Perfusion Two air pump (8 in Fig. 4 connects to 1 in Fig. 3) via another Y-connector. On the other stopper, a short piece of tubing is attached to the unused side of the Y-connector for use as a pressure relief valve while KRC and fixative are gassed with O₂/CO₂ (2a in Fig. 6 and 5 in Fig. 4). This pressure relief tubing is folded over and clamped with a tubing clamp when pressure is applied by the air pump during the perfusion.

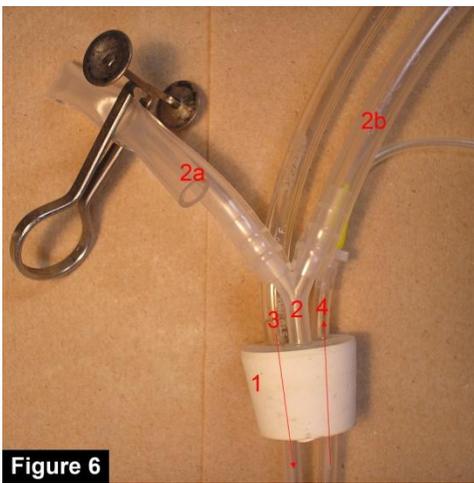


Figure 6

Connecting the perfusate bottles to the perfusion needle: Through the second hole of the bottle stoppers, run a piece of Tygon tubing (4 in Figs. 5 and 6; must be long enough to reach the bottom of the bottles), which is then connected to the three-way valve (3 in Fig. 4). The third connector on the three-way valve is connected via a luer-lock connector to IV tubing, to which the perfusion needle (4 in Fig. 4) is attached. A flow regulator/clamp is installed on this segment of the tubing. The length of the IV tubing from the bottles to valve and then the needle should be kept as short as practical: the longer the distance between the

water bath and the animal the greater the drop in temperature of the perfusates. (The temperature of the water bath may need to be adjusted in order to deliver fixative at 37°C, depending on the length of tubing, ambient air temperature, etc.)

Connecting the perfusate bottles to the oxygenation system: Through third hole in the stoppers, run a piece of Tygon tubing (3 in Figs. 5 and 6; long enough to reach the bottom of the bottles). Attach air stones (2 in Fig. 4) on the bottle side of the tubing. The other side of tubing is connected to O₂/CO₂ cylinder-regulator, with a clamp in between (1 in Fig. 4; clamp not shown).

The anesthesia system (Figs. 2, 7 and 8) consists of a halothane vaporizer driven by a O₂/CO₂ cylinder, a nose cone, endotracheal tube, and a ventilator to ventilate the anesthetized animal during the dissection and the initial stages of perfusion. Also, a large glass desiccator is used to anesthetize the animal prior to putting on the nose cone (an optional small Plexiglas chamber connected to the vaporizer can be used for this purpose).

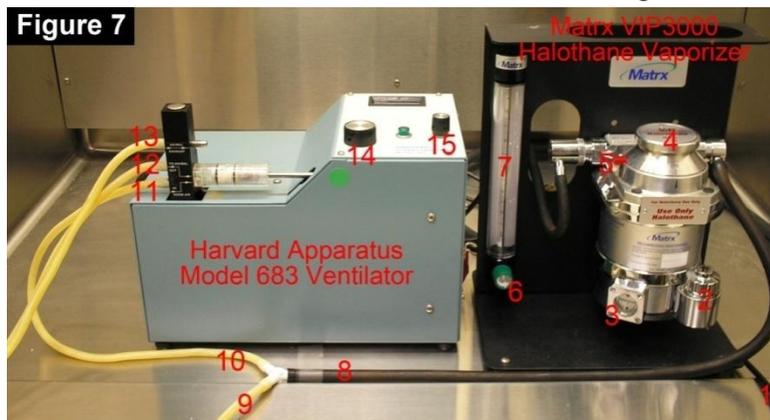


Figure 7

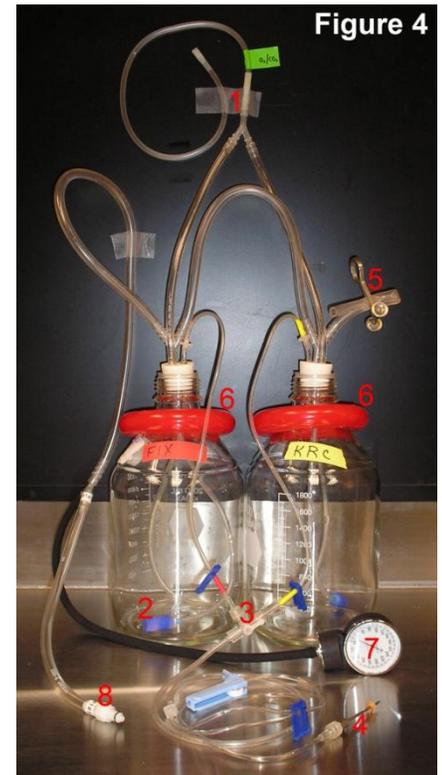


Figure 4

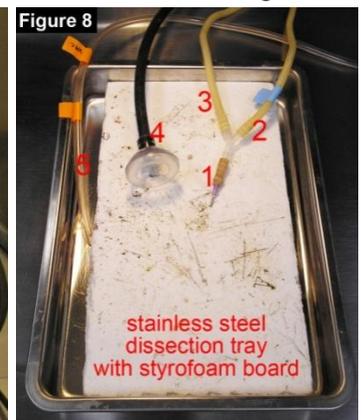
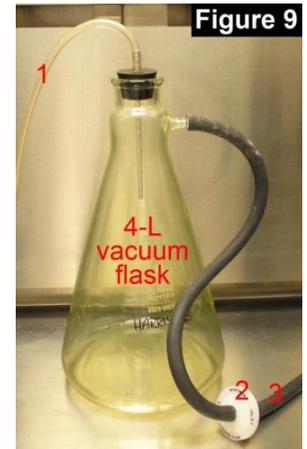


Figure 8

Connecting the oxygenation system to the anesthesia system: Connect O₂/CO₂ regulator to the O₂ input on the back panel of vaporizer (behind the O₂/CO₂ pressure control; 6 in Fig. 7). Connect the halothane output (8 in Fig. 7) to a Y-connector. One side of the Y-connector is attached to a piece of rubber tubing (9 in Fig. 7) leading to the nose cone (4 in Fig. 8). Use another piece of rubber tubing to connect the other side of the Y-connector to air intake of the ventilator (11 in Fig. 7). Output from the ventilator (12 in Fig. 7) is connected to the endotracheal tube (1 in Fig. 8; a blunted 16-gauge needle) via a Y-connector. Exhaust line from the endotracheal tube (2 in Fig. 8) connects back to the exhaust port of the ventilator (13 in Fig. 7). Make sure to label the exhaust line.

The waste collection system (Figs. 2 and 9) is used to remove blood and perfusate from the dissection tray as it accumulates, and consists of a 4-L vacuum flask attached to vacuum line. A #11½ one-hole stopper is used to seal the flask (Fig. 9). Through the hole, attach a short segment of a plastic serological pipet (long enough to pass the vacuum port of the flask), to which Tygon tubing is connected (1 in Fig. 9). Place the other end of this tubing to the dissection tray (5 in Fig. 8). The flask is connected to vacuum line via heavy rubber vacuum tube (3 in Fig. 9). An in-line filter (2 in Fig. 9) is placed to prevent waste liquid from getting into vacuum line. After the procedure the contents of the vacuum flask are transferred to appropriate disposal containers in accordance with local institutional policies.



3. Anesthesia

The objective is to anesthetize the animal so that it does not experience pain during the dissection but is still alive with the heart beating at the time of perfusion. Weigh the animal and record pertinent information on the Perfusion Record Sheet. A large desiccator with a wad of 4-5 small Kimwipes is used as an anesthesia chamber. Five minutes prior to starting, add 1.5 ml of 100% halothane to the Kimwipes below the perforated plate and put the top on the desiccator. Place the animal in the desiccator after the atmosphere has equilibrated for 5 minutes. Open the O₂/CO₂ regulator, set the vaporizer at 5% halothane (press the release button [5 in Fig. 7] and turn the dial control [4 in Fig. 7]), and adjust the O₂ flow to 400 ccm (6 [control] and 7 [indicator] in Fig. 7). Also turn the ventilator on, and adjust the stroke rate (15 in Fig. 7) to 120 breaths/min and the tidal volume (14 in Fig. 7) to 1.5 cc. In about 1.5 to 2 minutes the animal should begin to stumble and fall over. Anesthesia should have reached the stage where the animal is non-responsive to toe pinch in about 2.5-3 minutes. When the animal stops trying to stand up, place the animal on the dissection tray with its nose inside the nose cone. Tape or pin the animal to the Styrofoam board. Begin the surgery when the animal is non-responsive to toe pinch.

If the Plexiglas rodent anesthesia chamber is used instead of the desiccator, place the animal in the chamber attached to the halothane vaporizer. Set the vaporizer at 5% halothane and adjust the O₂ flow to 400 ccm. The rat should become anesthetized in about 4 minutes. Check this by rotating the chamber slightly and observing if the animal tries to adjust its stance, i.e., tries to stay on its feet. It is preferable that the vaporizer be connected separately to the box, nose cone and ventilator using Y-connectors and tubing. If the nose cone is connected to the box via the box's exhaust as shown in some vendor's diagrams, the animal will not continuously receive the same level of halothane via the nose cone after the box is opened. In the absence of a halothane vaporizer, add 0.5 ml of halothane to one wadded up Kimwipe in the bottom of a 50 ml conical tube and use as a nose cone.

4. Dissection and Perfusion

This procedure is best performed by a "surgeon" and "assistant." The assistant has a crucial role in preparing the perfusion apparatus while the surgeon is anesthetizing the animal and performing the dissection, and in regulating the perfusion pressure and changing from KRC to fixative in the early stages of the perfusion. After the heart is sufficiently fixed to hold the needle in place, the rest of the perfusion could be performed alone by either person. In the Step-By-Step procedure outline below, the minimal role of the assistant is shown in *italics*.

Before the animal is anesthetized, the perfusion apparatus must be completely pressure-tested, the perfusion solutions brought to 41°C, and KRC gassed with O₂/CO₂ (95%/5%). When the dissection begins, the assistant must flush fixative through the entire system to remove air bubbles, and then KRC should be flushed through the system. As the surgeon begins the dissection, the assistant must get the pressure stabilized at 80 mmHg and be ready to begin the flow

of the prefix to allow about 10 ml (enough to clear the tubing and begin perfusing with warm buffer) before the surgeon inserts the perfusion needle into the heart. The assistant must be ready to switch from KRC to fixative within 5 sec after the left ventricle is penetrated and KRC flush started. The assistant will change the pressure as required to ensure good fixation at least as long as the surgeon is tending to the animal.

5. Step-By-Step Procedure:

All procedures are performed in a well-ventilated fume hood or on a necropsy table with down draft. Appropriate protective clothing, including gloves and eye protection, are worn. All toxic waste is appropriately contained and disposed of in approved manner.

The minimal role of the assistant is shown in *italics*.

1. After filling the perfusate bottles, open the pressure release clamp, and close three-way valve and flow regulator on the perfusate line.
2. Connect Tygon tubing from the perfusate bottles labeled "#1" to the #1 port on Perfusion Two pump.
3. On Perfusion Two pump, set Air Tank switch at "Disconnect" and Perfusion Pressure at "Hold" positions. Adjust air pressure control dial to 80 mmHg.
4. Warm the perfusate in water bath to 41°C, and oxygenize it for 30 min.
5. Close pressure release clamp on perfusate bottles.
6. Anesthetize the animal in a large dessicator with a wad of 4-5 small Kimwipes. Five minutes prior to starting, add 1.5 ml of halothane to the Kimwipes below the perforated plate and put the top on the dessicator.
7. Start the vaporizer set at 5% halothane and the O₂ flow at 400 ccm, and the ventilator running at 120 breaths/min and a tidal volume of 1.5 cc.
8. *While the animal is being anesthetized:*
 - i. *Turn on the vacuum for the waste collection system.*
 - ii. *The O₂/CO₂ supply to the perfusate bottles must be closed and tightly clamped.*
 - iii. *The pressure release tubing must be clamped tightly.*
 - iv. *Turn on Perfusion Two pump (the main switch is on the back panel). Compressor will start pumping air until the pressure reaches about 80 mmHg in air tank (This will not be indicated on sphygmomanometer, which measures the pressure in perfusate bottles). Turn Air Tank switch to "Connect" and Perfusion Pressure switch to "Release" positions. Adjust air pressure to 80 mmHg (Always check with the sphygmomanometer - the indicator on the pressure control dial is approximate). Pressure can be released by opening the valve on hand pump.*
 - v. *Open the three-way valve and flow regulator to flush the perfusate system, first with fixative and then the KRC, to remove all air bubbles from the tubing. The waste fluid is drained into the dissection tray. Once this is done, close the flow regulator, with three-way valve open to KRC.*
9. Remove the animal from chamber and place on the dissection tray and place its nose in the nose cone.
10. Test the animal's anesthesia level with a toe pinch. A sufficiently anesthetized animal will not respond.
11. Make a chin-to-sternum incision.
12. Isolate the trachea, elevate the trachea by placing the tip of a pair of curved hemostats under it to place a piece of silk suture (or cotton tread) around it proximal to the larynx and tie a loose knot in it.
13. Make a lateral cut across trachea just below larynx and above the suture, being careful not to cut completely through trachea or to let any fluids get in it.
14. Reduce the O₂ flow to halothane vaporizer 100 ccm and reduce the halothane level to 4%.
15. Remove the nose cone.
16. Immediately insert the endotracheal tube into the trachea and tie the suture. Secure the tube by taping it to a rolled up paper towel just above the animal's head.
17. Clamp the exhaust line of the endotracheal tube or place your right index finger over the exhaust port on the ventilator for 2-3 breaths to create an artificial "sigh."
18. Open the chest by making a small hole below sternum and carefully cut laterally just under the diaphragm. Then carefully cut the diaphragm along the ribcage. Cut up the two sides of the chest, keeping the scissors away from the heart and lungs, until the incisions reaches just below the forelimbs. Clamp the sternum with a pair of hemostats and lift it up to expose the chest cavity.

19. Clean away the pleura to release the heart and lungs and repeat the artificial sigh sequence.
20. *The assistant confirms the perfusion system has a pressure of 80 mmHg, flushes the system with enough fixative to ensure warm fix will be delivered, switches to KRC to clear the tubing of fixative and ensure warm KRC will be delivered. The waste is collected as above.*
21. With artificial respiration complete (heart beating and lungs moving in and out) prime the perfusion system.
22. Confirm that the assistant has the perfusion system ready to go, then make an incision with iris scissors in the right ventricle and ask your assistant to turn on KRC.
23. Take hold the apex of the heart with your thumb and forefinger or a good pair of tissue forceps. With the warm KRC flowing at 80 mmHg, insert the perfusion needle into left ventricle until it stops at the cork disk and hold it there. KRC will flush the blood through the systemic circulation and out the right ventricle. There should be no fluid entering the endotracheal tube and ventilator tubing.
24. In 3-5 seconds have the assistant switch the 3-way valve to begin the flow of fixative and increase the pressure to 120 mmHg.
25. After 15 seconds gradually increase the pressure to 180 mmHg and maintain this pressure for 5 min (see step 23). During this time the animal should be having spasms and contractions and should start to stiffen. When the heart is fixed, usually within a couple of minutes, and the needle is secure the surgeon can let go of it, or if need be, prop it up so that it won't move.
26. Turn off the ventilator and vaporizer and remove the endotracheal tube.
27. Clip the chin and observe fixative oozing from the chin. Other signs of a good perfusion are feet and tail are pale white and the animal's tail is stiff down to its tip.
28. Decrease the perfusion pressure to 80 mmHg by opening the valve on hand pump, and adjust the pressure control dial to maintain the pressure at 80 mmHg. Maintain this pressure for about 50 min. At the end of this period, approximately 1800 ml of fixative should have been used. **DO NOT ALLOW THE FIXATIVE BOTTLE TO RUN OUT OF FIXATIVE BECAUSE THAT COULD CAUSE AIR TO BE PERFUSED INTO THE BRAIN.** Reduce the flow of fixative even to the point of cutting it off, if necessary, but the time the brain is fixed should be at least 60 min. Note that the minimum pressure adjustable by Perfusion Two is about 70 mmHg. If it must be reduced further, either 1) flick the Perfusion Pressure switch with Air Tank switch at "Connect" position, or 2) turn off Perfusion Two (with the Air Tank switch at "Connect" and Perfusion Pressure switch at "Release") and use the hand pump.
29. OPTIONAL: Switch the 3-way valve to perfuse with Krebs-Ringer Carbicarb at 80 mmHg for 15 min to wash with about 400 ml of buffer.
30. OPTIONAL: Reduce the pressure to 30-40 mmHg and allow an additional liter of buffer to perfuse through over the next 45 min. **DO NOT ALLOW THE KREBS-RINGER CARBICARB BOTTLE TO RUN OUT BECAUSE THAT COULD CAUSE AIR TO BE PERFUSED INTO THE BRAIN.**
31. Disconnect the perfusion needle and remove the animal from the Styrofoam board.
32. Turn off Perfusion Two pump, release remaining pressure through vent on perfusate bottles, discard the remaining perfusate into waste containers, rinse the perfusate bottles with diH₂O, flush the perfusate line with diH₂O.
33. Remove the head by cutting through the neck with a sharp knife and dissect out the brain from the skull being careful not to nick it with tools. Place the brain in a container filled with the fixative until it is sectioned (or shipped).
34. Examine the brain under a dissecting microscope to determine whether or not small red veins are visible. A well-perfused brain will appear light gold and have no red veins whereas a poorly perfused will have a pink tinge.
35. If the post-fixation wash was effective, the brain should not have an aldehyde odor.
36. If the surface of the brain appears to be well perfused, dissect the area(s) of interest and place them in 0.1 M sodium cacodylate for further processing or shipment.