Purpose: The goal of perfusion fixation is to use the vascular system of a deeply anesthetized animal to deliver fixatives to the tissues of interest. This is the optimal method of tissue preservation because the tissues are fixed before autolysis begins. Perfused tissues are less susceptible to artifacts caused by handling. Techniques for fixation vary depending on the organ and the desired processing. Appropriate literature should be consulted to determine the ideal technique for the organ of concern. The following technique is appropriate for harvesting brain and organs with circulation supplied by the left side of the heart. This method combines tissue fixation with euthanasia and can only be performed as a terminal procedure.

SCOPE: This procedure applies to all CMLAF technicians and all principal investigators and associated staff. The above individuals will have completed rodent module training and will perform procedures according to an approved protocol.

1.0 Procedure:
A. Supplies:
   a. 2 empty 250ml fluid bags with fluid lines attached (see diagram)
   b. 60ml syringe and large bore needle
   c. IV stand
   d. Butterfly catheter (23g) with the needle blunted
   e. Bandage tape
   f. Mosquito hemostats
   g. Small scissors
   h. Jewelers forceps or equivalent (see diagram)
   i. Scalpel handle and #10 blade
   j. Alm retractor (see diagram)
   k. Glass pan to catch waste fluids
   l. Freshly made 4% paraformaldehyde (PFA) 10-150ml per mouse
   m. 0.9% saline (or preferred flush) 8-25ml per mouse
   n. Anesthetic (as per protocol)
   o. Chemical fume hood

B. Set Up
   a. 4% PFA must be made fresh on the day of the procedure in a chemical fume hood.
   b. The perfusion process should be performed in a chemical fume hood for the best personal protection. Perfusion can be performed in a well ventilated area if a chemical fume hood is not available.
c. Fill one IV bag with the fresh 4% PFA using a 60 ml syringe and a large bore needle
d. Fill the other IV bag with the flush
e. Set up the IV lines in a “piggy back” fashion (see diagram) with the blunt butterfly needle attached to the end
f. Hang the bags from the IV pole at least 30 cm, but not more than 120 cm above the animal
g. Flush any air bubbles out of the IV line
h. Use the saline to flush any fixative out of the line before starting

C. Anesthesia
a. Barbiturates like pentobarbital (100mg/kg IP) provide the best anesthesia for perfusion
b. Pentobarbital should be diluted to 15mg/ml by adding 0.3ml undiluted pentobarbital (50mg/ml) to 0.7ml sterile saline (1ml total). This dilution will yield a dose of 0.2ml for a 30 gram mouse.
c. Administer the anesthetic to the mouse and allow it to rest in a cage by itself in a dark, quiet environment
d. The withdrawal reflex must be absent in each pelvic limb before the perfusion can begin.

D. Perfusion
a. Place the mouse on its back and tape each limb down to the glass pan
b. Check the withdrawal reflex once more to assure adequate depth of anesthesia
c. Make a midline skin incision from the thoracic inlet to the pelvis
d. Use scissors to carefully open the abdomen and expose the liver and intestines
e. Grasp the tip of the sternum (xyphoid process) with forceps and make a 1cm incision down the midline of the sternum (too large an incision risks cutting the major vessels as they enter the thoracic inlet)
f. Place the Alm retractor into the chest incision and adjust the knob until the sternum is held open widely enough to visualize the heart
g. Grasp the heart gently by the right ventricle and lift it to the midline and slightly out of the chest
h. Use the scissors to make a small nick in the apex of the left ventricle (see diagram) The left ventricle is thicker and lighter pink than the right ventricle.
i. Place the blunt butterfly needle into the heart incision toward the aorta and clamp in place with hemostats
j. Start the flow of the flush and watch the chamber on the IV line to assure that the fluid is dripping
k. Use scissors to cut the right atrium to allow the perfusate to exit the circulation
l. When the fluid exiting the mouse is clear of blood, close the flush line and open the 4% PFA line.
m. Muscle contractions and blanching of the liver and mesenteric blood vessels are signs of good perfusion.
n. Perfusion is complete when all muscle contractions have stopped, the liver and mesenteric vessels are blanched and the desired amount of preservative has passed through the circulatory system. The mouse should be stiff.
o. PFA and other fixatives must be collected after the perfusion and stored appropriately as hazardous chemical waste. Contact Environmental Health and Safety at 829-3301 for disposal procedures.

E. Troubleshooting
   a. Fluid dripping from the mouse’s nose
      1. Indicates too much fluid pressure
      2. Lower the height of the IV bag until the fluid stops dripping from the nose
   b. Fluid is not seen dripping in the IV chamber
      1. Indicates that the fluid is not flowing into the mouse’s circulatory system
      2. Close the fluid line, reposition the butterfly needle, reclamp and reopen the fluid line
   c. Mouse still has a positive withdrawal reflex before the procedure
      1. Indicates inadequate anesthesia
      2. Administer 1/3 the original dose of pentobarbital and allow the mouse to rest in a dark, quiet cage until withdrawal reflexes are absent
      3. Overdose of the pentobarbital will not negatively affect the perfusion as the procedure can also be performed if the mouse is freshly euthanized.

F. Conclusion: When performed properly, whole body fixation perfusion is a humane method for ideal tissue preservation in the mouse.

Figure 1. Alm retractor. This instrument is placed in the incision to hold the edges of the sternum apart. The instrument is opened by turning the knob at the closed end.
Figure 2. Jewelers forceps. These forceps are small enough to grasp the heart within the confined space of the mouse chest. These are also made with curved tips. Any small, atraumatic forceps will work.

Figure 3. In Situ anatomy of the mouse heart. Black arrow indicates location of the butterfly catheter insertion site. Light blue arrow indicates the position of the right auricle that is cut to allow the perfusate to drain.
Figure 4. Example of a commercially available "piggy back" set up for perfusion. A "piggy back" set up can also be made out of 2 regular IV lines by attaching a large bore needle to the end of one of the IV lines and inserting the needle into the injection port (see photo) of the other line.

Figure 5. Parts of an IV set up.