Millar Protocol

N. LaCross 2003

General Points:

- The whole procedure should take around 3 hours from start to finish. It can be less if things go well, and more if they don't. Plan accordingly.
- Be sure that all required equipment and supplies are available and close at hand. Be doubly sure to check the oxygen and isofluorane levels.
- Be conscious of where the catheter is *at all times*, and take great care to handle it gently. Above all, NEVER touch the tip with anything other than the edge of a kimwipe.

One Final Note on Catheter Durability:

For its extremely small size, the Millar catheter is quite durable and should provide many uses (at least 50+) with proper care and maintenance. However, this does not mean that the catheter will be able to survive repeated abuse. Following the guidelines below should maximize the catheter's lifespan and performance.

1. When grasping the catheter, always hold it at least 1 cm below the last electrode with your fingertips only (never your fingernails or forceps)
2. Never touch the catheter tip (electrodes and pressure transducer)
3. Avoid bending the catheter to any great degree, especially the tip
4. Avoid applying sudden, large pressure changes to the tip, as this can damage the transducer
5. Only use the catheter with the proper equipment and solutions (ddH_2O, 0.9% saline, alconox, and terg-a-zyme)
6. Always rinse the catheter tip in ddH_2O and gently dry with a kimwipe after cleaning so a deposit doesn't form.

Equipment:

All equipment should already be in room 7607

- Two curved tip, size 5/45 forceps
- One straight tip, size 5 forceps
- One pair of curved tip scissors
- One cautery with spare tips
- Several glass "occluders." These are basically bent and melted glass pipets, and are very simple to make. Take some of the borosilicate glass tubes, melt one end closed, and then heat them about 1cm from that end to make a 80-90° bend.
- Clear plastic block with a series of sequentially larger holes drilled in it. This is important, and should be on the desk.
- Thermocouple temp. probe and thermometer (this also should be on the desk, but it does like to wander away…)

- Kimwipes (these are your multi-purpose best friends. You'll use a LOT of these)
- Cotton tip applicators (you'll use many of these, too)
- a spool of 3-0 suture thread
- superglue
- Tubing for 30 gauge needle
- 20, 25, and 30 gauge needles (you'll need a few of each, so make sure you have a box of each handy)
- 20 gauge tubing adaptors (basically short, blunt tipped needles)
- 1mL insulin syringes
- 10 mL syringes
- 0.9% saline (sterile filtered) and 20% saline (unfiltered)
- 1% Terg-a-zyme
- 70% ethanol
- Isofluorane
- a bottle of ddH_2O
- Heparin 1000 U/mL (from hospital pharmacy)
- Dobutamine 25 mg/20 mL (from hospital pharmacy)
- Propranalol 10 mg/mL (from hospital pharmacy)
- 25% human serum albumin (from hospital pharmacy)

Setup:

The surgical setup is located in room 7607 of Med. Sci. II. The room has a combination lock; see the sheet on the door for contact and access information.

- The Millar catheter should be stored in its box in the bottom right drawer of the desk. Carefully take it out of the box, place the tip in a 10mL syringe of 0.9% saline, and plug in the two connectors to the appropriate wires. You may need to gently prop the wire in place so that the catheter does not slide out of the syringe.
- Turn on the Aria box, which is in the top rightmost drawer (this is what the catheter is plugged in to). The power switch is located on the front side. Look at the conductance dial. When the catheter is in a large volume (1mL +) of saline, the conductance dial should be maxed out (i.e. the needle should be all the way to the left). Sometimes this can take a minute or two if the electronics need to warm up. If the dial is not maxed out, there is a problem; see the troubleshooting section.
- The catheter and Aria control unit must be left on for at least 30 minutes. This allows the electronics to warm up and should prevent pressure drift. In the meantime, the rest of the setup procedure can be completed.
- Turn on the computer, and start Biobench when it's done booting up. In the file menu, go the logging option and change the file name. It seems to work best when it's in the format of a letter, two numbers, and hyphen,

another number and then two letters (ex: E16-5tm). Change the scale on the x-y graph to something reasonable (ex: 0 to 1 y axis, -3 to -1 x axis).

- When the box and catheter have been powered up for at least 30 minutes, go to the calibration dialog (go to the configure menu, select channel, then select calibrate). Turn the knob on the Aria box from subject to calibrate, then turn the Relative Volume Units knob to 5 RVU. Click on the button in Biobench near where it says 5 RVU to lock in the appropriate value. Turn the RVU knob to 45, and click on the button where it says 45 RVU. Go back to the data acquisition screen.
- Turn on the heating pad to the first setting, and leave it there. While this won't be enough to keep the mouse at exactly 37°C, it will help, and higher settings will often overheat the mouse, which is a bad thing. It is simple to warm the mouse a little; its much more difficult to cool it down.
- Inspect the surgical tools and plexiglass plate for cleanliness. Wipe them down with 70% EtOH if they appear dirty. Since this is a terminal procedure, sterility is not a requirement, but clean implements will make your job easier.
- Check the latex of the mask for tears and degradation. Replace if necessary. To do this, simply cut a piece of latex from a glove and place over the end of the tube. Slide the zip tie in place to secure it, and cut away the excess latex. Cut a small hole near the bottom for the mouse's nose.
- Briefly turn on the ventilator and check the settings. Put a 20 gauge tubing adaptor on the tube from the ventilator, beveled side up. Breath rate should be 120/min, the peak pressure when the tubing adaptor hole is closed off should be ~20, and the peak volume level should be low (i.e. the bead in the regulator should only get 3-4 mm from the bottom).
- Prepare 4 10mL syringes: 2 with 0.9% saline (label one rinse saline and the other 0.9% saline), 1 with 1% Terg-a-zyme, and 1 with ddH_2O. These can be reused for several experiments.
- Double check the O_2 and isofluorane levels. Refill/replace as necessary.

<u>Solutions:</u>

- Using a 10mL syringe and a 20 gauge (or smaller) needle, draw out 1mL of 25% human serum albumin. Currently, this is located in fridge in room 7720. To this add 1.5mL 0.9% saline. The albumin is very viscous, so be sure to mix thoroughly. When mixed, fit a 30 gauge needle to the syringe and eliminate all air bubbles.
- Now place the syringe with the albumin solution into a syringe pump, but don't turn it on yet. Cut a length of tubing (say a foot or so) and slide one end onto the 30 gauge needle of the syringe in the pump.
- Take another 30 gauge needle, and use needle-nose pliers to carefully crush the plastic base (taking care not to bend the metal needle itself). The actual metal needle should become loose, and you'll eventually be

able to pull it out. Slide the blunt part of the needle into the free end of the tube attached to the syringe pump. You've just made yourself an IV. The albumin solution dries quickly and can plug the needle, so don't start the syringe pump until just before you plan to use it.

- Now you need to make the dobutamine solution. Take another 10mL syringe and fill it with 4.95mL of 0.9% saline. To this add 50uL of dobutamine (use an insulin syringe). The dobutamine and propranalol are stored in a pipet tip box covered in aluminum foil. These reagents are photosensitive (hence the foil), so put the stocks back in darkness when finished.
- Finally, take two more insulin syringes. Fill one with 100uL of heparin (located in the top left drawer), and the other with ~10uL of propranalol (in a microfuge tube next to the dobutamine). It can be difficult to obtain such a small volume with any accuracy, but try to get as close as possible. Overestimation is preferable to underestimation in this case; too much propranalol can easily kill the mouse.
- Check O_2 and isofluorane levels one final time (are you seeing a trend here?)

Surgery:

A Note on Isofluorane:

Isofluorane is a fast acting, effective anesthetic whose primary virtue is a relative lack of effect on hemodynamic performance. However, its half-life is extremely short, meaning that if a mouse is taken off of it, it will begin to wake up in about 30-40 seconds. Therefore, make sure that the mouse has a supply of isofluorane that is interrupted for no more than 20 seconds at a time.

Preliminary Stuff:

- Open the valves on the O_2 tank completely. Turn the regulator on the isofluorane atomizer to 0.6L/min, and set the isofluorane level at 2-2.5%. Check the tubing connector to make sure that the anesthesia is going to the chamber (only first set of valves should be open).
- Get the mouse and place him in the chamber. Be sure that the plug is flush to the walls forming a good seal. Turn on the anesthesia to the mask at this point (the second set of valves).
- After a few moments, you should notice the mouse stumbling around and generally acting loopy. Wait until the mouse is completely under before you remove it from the chamber (usually 5-10 minutes). Try moving the chamber around some to disturb the mouse if you're not sure. If it's still awake, it usually starts moving a little.
- When you're sure its down, remove the mouse and place it on its back in the center of the plexiglass plate. Place the mouse's nose in the hole in the latex of the mask. Tape down the mask tube. Mice are

obligate nose breathers, so it should stay under. Turn off the anesthesia to the chamber.

- Gently pinch the mouse's feet. If it doesn't react, it's fully under.
- Spread the mouse's limbs and tape them down. Carefully insert the rectal temp probe and turn the thermometer on. It should go in quite easily, so don't force it. Physiological temperature is 37°C, but the anesthesia often cools the mice down a little. If the mouse is cold, use the incandescent lamp to warm it up a little. You will have to monitor the temp very carefully, as it is easy to overheat the mouse. This is bad. Its much better to have the mouse be too cold than too warm. **Continually monitor the mouse's temp and adjust accordingly.**
- Apply some Nair to the mouse's chest and spread it around with a cotton tip applicator. You'll need to use a fair amount, which is unfortunate since this stuff smells awful. The idea is to remove the hair from the areas where you'll be working. Wait for a few minutes.
- Take a cotton tip applicator and gently try to rub off some of the fur under the Nair. If it comes away easily, then it's done. Use more applicators and kimwipes to remove the rest of the fur and Nair. Soak a kimwipe in EtOH and wipe down the now bare skin to remove the last of the residue.

<u>Tracheal Cannulation:</u>

- Have a 20 gauge needle and a short (~2 inches) length of suture ready.
- Use the scissors to cut away the skin on the throat from the top of the chest to the bottom of the jaw. Take care not to puncture any major vessels. If minor bleeding occurs, use the cautery (sparingly) to stop it. The goal here is to expose the parotid glands, under which are the trachea and jugular veins.
- Using the forceps to bluntly dissect the parotid glands away from the trachea. There are two and they're loosely attached by connective tissue. Gently move them aside so they don't interfere with the surgery.
- You should now be able to see the muscle sheath that covers the trachea. The sheath is actually divided down the middle, so use the forceps to bluntly dissect it apart to expose the trachea. It will appear white with slightly raised cartilage rings. VERY carefully dissect under it with the forceps, and then gently pull the length of suture under the trachea. Great care must be taken since the carotid arteries are just underneath the trachea, and the experiment will be over rather quickly if you puncture one.
- Turn the anesthesia to the ventilator on, and switch the power back on.
- Pulling on the suture gently to expose the trachea fully, use the 20 gauge needle to poke a hole between cartilage rings.
- Quickly remove the mask and move it out of the way, and insert the tubing adaptor from the ventilator into the hole in the trachea. It needs to be in far enough not to fall out, but not too far (~1cm or so). If the chest rises and falls in time with the ventilator, the cannulation was successful. If it

doesn't, move the tube around until the chest moves. Replace the mask over the nose briefly if necessary.

- When a good position has been found, tie a knot in the suture around both the trachea and the tube inside it. Make sure you don't tie the knot below the end of the tube, or the mouse will be unable to breathe. Add a drop of superglue to the junction of the tube and trachea to make an airtight seal.
- Move the mouse's head to your left, allowing the ventilator tube to lie flat. This also allows better access to the jugular. You can switch sides if it makes things easier; just remember to reverse all directions that follow.
- Tape down the mouse's head, stretching the neck and exposing the jugular a little more. Also, tape down the ventilator tube and the plexiglass plate.

Jugular Cannulation:

- Turn on the syringe pump with the albumin solution and set it to 46 uL/min. Set the needle end onto a folded kimwipe. Before you insert the IV, you must make sure that all air bubbles are eliminated.
- Using the forceps, bluntly dissect the connective tissue away from the mouse's right jugular (it'll be on your left). Be extremely careful when working near the jugular. The vein wall is quite thin and therefore easy to damage. You don't need to clear everything off the jugular, just clear enough away to see what's going on.
- When a steady flow of albumin solution is coming out, pause the pump. Move the albumin syringe pump closer to the mouse so that there will be enough slack in the IV tube. Make sure that all air bubbles have been eliminated from the IV and the syringe.
- Grasp the end of the metal IV needle with one set of forceps (it will probably be easiest in your "dominant" hand) and gently hold some tissue just above the jugular with the other set. DO NOT grasp the jugular itself or you'll be doomed.
- While gently pulling up on the tissue to induce tension in the vein, carefully slide the IV needle a few millimeters (~4-5) into the jugular. CAREFULLY let go of the tissue and needle with the forceps. If you jerk them away, you'll probably either pull the needle out of the jugular, which is bad, or push it all the way through, which is worse.
- Turn on the syringe pump (it should still be at 46uL/min) for around 2 minutes. You want to infuse an initial bolus of 150-200uL. You should be able to see the flow in the vein. Try to move the needle around (gently) so that the end isn't pressed against the jugular wall. Ideally, the needle tip would be in the center of the vein. When a good position has been achieved, tape down the tube between the pump and the mouse, and add a drop of superglue to the upper part of the needle to hold everything in place.
- After the initial bolus, turn down the syringe pump to 6uL/min.

Thoracotomy:

- Use the scissors to make and incision in the skin of the chest 1-1.5cm to the left of the sternum, from the bottom to the top of the rib cage. Use the cautery to stop any bleeding.
- Make another incision from left to right just below the last ribs, and pull the flap of skin to the right, exposing the chest muscles. The surgical area should now be free of skin.
- Prepare a 25 gauge needle for making the stab wound in the left ventricle (LV). Basically, take a piece of tape, roll it up, and stuff it into the base of the needle using pliers to get a tight seal. This is to minimize blood loss by preventing it from running up the needle.

A Note on Cauterization:

Now you'll be putting the scissors away in favor of the cautery. This is primarily to eliminate as much loss of blood as possible, which it does admirably, but it also creates new problems in the process. The heat that cauterizes blood vessels can also severely damage delicate organs if proper care is not taken. This is especially true of the lungs and heart. Some heat damage to the lungs is often unavoidable as they lie close to the chest wall, but the damage should be minimized as much as possible. Extreme care should be taken when working near the heart, and there are no circumstances where damage cannot be avoided. A careless slip with the cautery can easily damage the heart beyond any repair as far as useful hemodynamic data is concerned.

- If you examine the right side of the chest (mouse's left), you should observe what appears to be a slight, localized vibration. This is the heart beating. Note the lowest point at which the vibration can still be seen; this is where the incision will be made.
- Using the cautery, gently begin to make an incision starting from the sternum and moving right. It is usually easiest to burn through the muscle layer first, thus exposing the ribs.
- Once the ribs are uncovered, use the forceps to grab one or the adjacent muscle and lift the rib cage away from the lungs. The idea is to limit damage to the lungs. Use the cautery to cut through the ribs, working your way from the sternum down the right side. You should be able to see the apex of the heart above your incision.
- Make certain that the incision is on the correct side of the diaphragm. Ideally, the incision will be just inside the chest cavity.
- If you examine the heart, you should be able to see some whitish connective tissue covering it. This is the pericardium. It can often make inserting the catheter much more difficult, so the next step is to remove it. Use the forceps to gently pull it away from the heart, making sure you don't grab the heart itself by mistake. Use kimwipes to soak up any blood.

- Now you're ready to insert the catheter into the LV. First, begin acquiring data from the catheter. Remove the tip from the syringe of saline, taking great care not to let it hit anything. Grasp the catheter in your right hand a centimeter or so from the tip, and hold the 25 gauge needle in your left. Carefully make a stab wound in the apex of the heart with the needle. Try to go in as straightly as possible; this should go right into the LV. Pull the needle out, and as quickly as possible insert the catheter into the hole. Be sure not to flex the catheter tip too much.
- The tip should be in just far enough so the last electrode is not visible. Catheter position has a large effect on the volume trace (not so much on pressure), so watch the computer screen to see how moving the catheter affects the trace. You should see nice, consistent waveforms for both volume and pressure, and the x-y plot should look vaguely like a rectangle. If the volume waves look flat or aren't there at all, check to make sure the last electrode is in the LV. If the pressure waves don't go down to near zero at the bottom, the catheter is probably in too far and is picking up arterial pressure. Usually, the max pressure is around 100mmHg, give or take 10. If the pressure spikes up much higher, especially on dobutamine, this can indicate that the heart wall is pressing on the transducer. Try moving the catheter to alleviate the problem.
- Use a spool of suture to prop the catheter in place. Eventually, a clot will form around the catheter and hold it in place.

<u>Baseline Data:</u>

- Check the temperature to ascertain the mouse is at or very near 37°C.
- When the traces have stabilized, begin to record baseline data. Do this by checking the log data box. If the log data box is unchecked while Biobench is acquiring data, it will continue to record data to that same file when the box is rechecked. Biobench will continue to record data to the same file until the stop button is hit. Every time you're finished recording data to a particular file, click the stop button and uncheck the log data box.
- In general, a data file should be between 10 and 30 seconds long. You cannot analyze more than 1.999 seconds of data, and files can be confusing if they are too long.
- In many cases, the movement of the lungs can introduce artifacts and waveform variations that are not actually part of the mouse's physiology. Try hitting the sigh breath switch for a few seconds while recording data. Sometimes it helps, other times not so much.
- For baseline data, record 1 or 2 files of normal waveforms, one file of occlusions (usually 2 or 3 occlusions in a file), and then another file of normal waveforms.
- An occlusion refers to a compression of the inferior vena cava (IVC). The IVC is very easy to find; it's the large vein that runs from the lower body through the diaphragm and between the lungs to the heart. Use a bent glass pipet to press down on it gently. This prevents blood from returning

to the heart, and you should see volume and pressure decreasing in a fairly linear fashion.
- Some normal numbers: max pressure around 100mmHg, + derivative around 10,000, and – derivative around –9,000.

Dobutamine Data:
- Remove the syringe pump containing albumin from the IV and connect the pump with dobutamine. Eliminate all air bubbles from the syringe before connecting it. Turn the pump to 10uL/min.
- Wait for the dobutamine to take effect. This usually takes 5-10 minutes. When it has taken effect, the heart rate will increase and the pressure-volume loops tend to widen and shift to the left. It's usually pretty obvious.
- Wait for the traces to stabilize. Record a file of normal waves, then a file of occlusions (2 or 3 in the file), and finally one more file of normal waves.
- Typical numbers seen with dobutamine: little or no change in max pressure, an increase in heart rate, + derivative between 12,000 and 18,000, and – derivative slightly lower than baseline (~ -8,000).
- Turn off the syringe pump.

Propranalol Data:
- You should already have a syringe with 10uL of propranalol and a syringe with 100uL of heparin prepared. Inject each intraperitoneally, just under the skin (try not to inject into an organ. It will take much longer to be effective).
- It usually takes 5-10 minutes for propranalol to take effect. When it does, both max pressure and heart rate will drop considerably, and the minimum pressure often increases a little. As with dobutamine, the effects are quite obvious.
- When the traces stabilize, record a file of normal waves, then a file of occlusions (2 or 3 in the file), and finally one more file of normal waves.
- If the dosage of propranalol was too high, it's easy for the heart rate and pressure to decrease to the point of endangering the mouse. If this seems to be happening, quickly record some data and then turn the dobutamine back on (a bit higher than before, if necessary; say 15-20uL/min).
- As soon as the data is recorded, turn the dobutamine back on (10uL/min). This should help counteract the effects of propranalol (a beta-blocker. Dobutamine is a beta-agonist). After 5-10 minutes, hemodynamic performance should start to increase, but it usually won't reach the levels previously seen under dobutamine (propranalol has a long half-life).
- It can be useful to record another file of normal traces after the dobutamine as taken effect. Its not needed for analysis, but can be helpful to see how the hemodynamics look before saline calibration.

Saline Calibration:

The Millar catheter doesn't measure volume directly; rather, it measures conductance, from which volume is later derived. However, the catheter also measures the conductance of the surrounding tissues in addition to the conductance of the blood; this skews the data and must be corrected for. Thus, the conductance signal is calibrated with hypertonic saline. The idea is to increase the conductance of the LV while keeping the actual volume the same, and from this deriving the number that must be subtracted from the absolute conductance to get the actual volume. When the saline is injected, the pressure-volume loops will widen and shift dramatically to the right.

- Fill an insulin syringe with 150-200uL of 20% saline. Take out another syringe and take off the caps, but leave it empty.
- Turn off the dobutamine pump, and remove the IV tube from the pump. Make sure that there are no air bubbles in the saline syringe, and slide the IV tube onto the needle.
- Start recording data.
- While watching the pressure-volume loops, *slowly* begin to inject the saline into the IV. All you want to do at this point is to fill the IV tube with saline, so when you start seeing effects on hemodynamics from the saline, stop injecting.
- Now you're ready to inject a small bolus of hypertonic saline into the left ventricle through the IV. You only need to inject a small amount (less than 50uL), so gently yet quickly push the syringe plunger in a bit. Injecting the saline rapidly yields the best results (you want a fast change in LV conductivity). However, an excess of hypertonic saline can easily kill the mouse, so care must be taken. You should see the pressure-volume loops widen and move quickly to the right, and then slowly move back.
- Do not inject a bolus of saline more than twice, or it can start to affect hemodynamics. In fact, only inject twice if the first one doesn't work out.
- When the injection is finished, draw the saline out of the IV tube with the syringe. It is a very small volume, so go slowly. When blood enters the tube and nears the syringe, remove the insulin syringe and hook the dobutamine pump back up. Turn on the pump to 10uL/min. This should limit the amount of saline entering the mouse, and also help restore hemodynamic performance.

<u>Volume Calibration:</u>

- When heart rate and pressure are back to something approaching normal, turn off the dobutamine pump and ready an empty insulin syringe.
- Place the rinse saline syringe near the mouse on the side with the catheter.
- Remove the catheter from the heart and carefully place the tip into the rinse saline syringe. Quickly insert the insulin syringe into the hole left by the catheter and collect as much blood as possible. You will need at least 300uL.

- Inject the blood into the holes in the plastic block, starting with the second to smallest and skipping every other one. There should be blood in four holes when finished. While recording data, take the catheter and insert the tip into each hole that has blood to measure the conductance. Measure each hole twice. If you are unable to get enough blood for all four holes, fill the first three, measure the conductance, and then take some of the blood to fill the largest hole. It is possible to do a 3-point calibration, but 4 points are considerably more accurate.
- Gently wipe off the catheter tip with the edge of a kimwipe. Place the catheter into the syringe of Terg-a-zyme for no less than 30 minutes. More cleaning time is never a bad idea.
- The mouse is usually dead from blood loss at this point. If it is an especially hardy specimen, euthanize it now. A simple, although messy, way is to snip the inferior vena cava.

Preparing the Heart:

- Cut the rib cage on either side of the chest to expose the heart. Carefully cut the heart away from the connecting vessels and out of the chest. Make certain that parts of the heart are not accidentally removed; it can be easy to mistake the atria for something that should be removed. It is best to cut far away from the heart and trim the extra tissue off later.
- Place the heart on a kimwipe and blot dry. Gently squeeze the heart to remove blood from the chambers. Carefully trim the extraneous tissue away. Again, don't remove the atria.
- Put the heart in a small weight boat filled with ddH_2O to rinse out the remainder of the blood. Gently squeeze the heart again until no more blood comes out.
- Thoroughly dry the heart again with a kimwipe, and weight it. It can now be crushed and frozen for western blot analysis, preserved for sectioning, etc.

Cleanup:

- Run the plastic block under hot water to wash the blood out. Use a syringe with water to get all of it if necessary. Dry the block, making sure that the holes are free of water.
- Turn off the ventilator, the isofluorane, the O_2 tank, the heating pad, the thermometer, and the syringe pumps. Place the mouse in a glove, tape it shut, and bring it down to the carcass cooler on the 3rd floor.
- Use kimwipes and 70% EtOH to clean the surgical tools and the plexiglass plate.
- Put the tubing adapter used for the tracheotomy, along with all used needles, in a sharps container. The syringes of albumin and dobutamine solutions can be used for up to two days; after that they should also be placed in a sharps container.

- Put all used disposable items (cotton tip applicators, kimwipes, etc) into the appropriate waste containers. All items with blood must go into a biohazard waste bucket.
- After at least 30 minutes has elapsed, remove the catheter from the cleaning solution and rinse it in the syringe of ddH_2O. Dry the tip with the edge of a kimwipe and put the catheter back into the box for storage.