# Dissection of Bullfrog Sacculus

**Items to have ready:**
MS-222 (tricaine methane sulfonate)
Dissection tools
35 mm dishes
1 X frog standard saline: add 90 mL of water to 10 ml of 10X frog standard saline (formulation at end of protocol); if possible, oxygenate the solution for about 10 min
Protease XXIV (optional; Sigma, Cat #P8038)

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**Anesthesia and Euthanasia of Bullfrogs:**

1. Anesthetize bullfrog by submersion in a solution containing 250 mg/L MS-222 (tricaine methane sulfonate) in water, pH-balanced to 7.0 with sodium bicarbonate. Check the level of anesthesia by monitoring eye-blink. Sacrifice animal following approved guidelines.
2. With sharp surgical scissors cut back from the mouth past the tympanum. Now cut across the spinal column to remove the palate and top of the head.

**Removal of inner ear from the head:**

1. Using a #10 scalpel blade, cut through the palate to reveal the underlying muscle (Figure 1A). This is best done by sliding the scalpel under the palate with the blade pointing up toward the ceiling and lifting your wrist up to cut through the palate.
2. Gently scrape away the muscle and connective tissue (Figures 1B and C). You should be able to see the faint hint of the white otoconia-filled inner ear encased within the cartilage. This can be done under the microscope if you prefer (Figure 1C
3. With your scalpel blade parallel to the benchtop, begin to gently shave away the cartilage to reveal the inner ear (Fig 2, panels 1-3). Each shave should be very shallow. Do not remove all of the cartilage in one large piece. The inner ear should be exposed over the course of about 7-10 shallow carvings. Make sure you have exposed enough of the area.

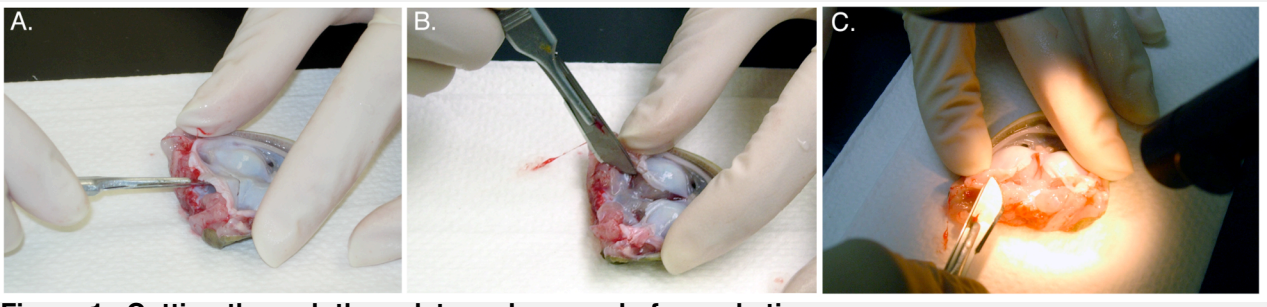


**Figure 1. Cutting through the palate and removal of muscle tissue**

4. Cut the bone in two places (Fig 2, panel 4, red arrows.) with your scalpel. These cuts should be perpendicular to the benchtop and depending upon the age of the animal may require some force.
5. Cut the semicircular canals and the nerves with your Vannas scissors. Grab one of the ampullae and gently pull while doing the cuts (ie., Fig 2, panel 6). These cuts are somewhat blind, although if you removed enough of the cartilage you may be able to see some of them. The nerve is going back toward midline (toward the spinal column) and is very white and thick
6. Gently grab part of the semicircular canals with your course forceps and very carefully start to lift the inner ear out of the head (Fig 2, panels 5-9). Keep your eyes open for things that need to be cut. Do not pull or tug on the tissue.
7. Place the inner ear into a 35 mm dish, containing 5 mls of frog standard saline.
8. Gently submerge the inner ear into the saline.

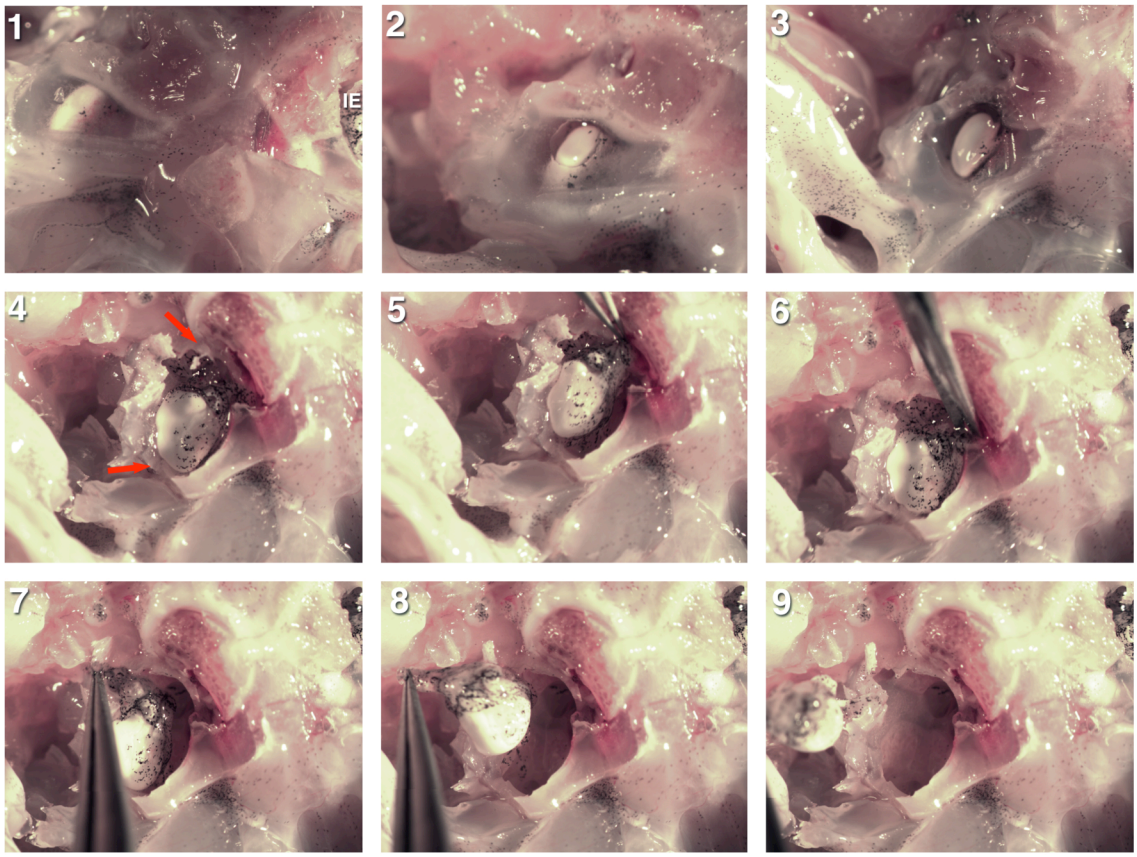


**Figure 3. Step by step images of removal of inner ear from the frog head.**

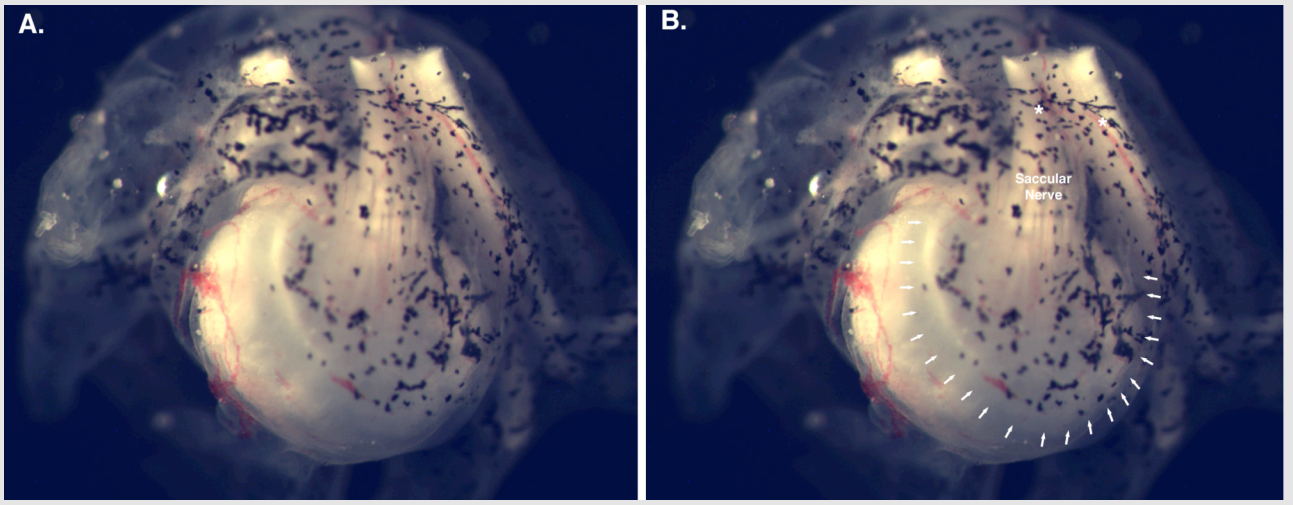


**Figure 3. Isolated inner ear.** Arrows in panel B designate the boundary of the saccular macula. Asterisks designate blood vessels that run along the nerves and can be used a landmarks.

**Dissection of the sacculus from the labrinyth:**

1. Orient the inner ear as depicted in Figure 3. You should see the severed nerves and the blood vessels that run along it (Fig 3B, asterisks). The margin of the saccular macula can be seen as an indentation (Fig 3B, arrows). Often it is easiest to see things if you reflect your light up from the bottom or parallel to the bottom of the dish.
2. First open the perilymphatic cistern and cut it away from the back of the sacculus. The perilymphatic cistern is the melanocyte-containing epithelium that overlies the back of the sacculus and the saccular nerve. This tissue is connected to the back of the sacculus by fibrous connections, which must be cut and not pulled on.
3. Once the perilymphatic cistern is cut away, dissect the saccular macula way from the rest of the inner ear by cutting at the edge of the saccular macula. Don't forget to cut underneath the nerve and to cut the nerves away from the other tissue. Try not to manipulate things too much or the otoconia leak out making it difficult to see.
4. Gently grab the saccular nerve and pull back the saccular macula. In doing so you will flip it over so that the nerve is now at the bottom of the dish.

*From this point on, the sacculus cannot cross the fluid-air interface or you will risk damaging the hair cells. All manipulations and solution exchanges must be done with the tissue under fluid. We use the blunt end of a pasteur pipet to move the tissue.*

5. Gently remove the otoconia-usually you can do this by sweeping them away gently with your forceps. Do not allow your forceps to touch the otolithic membrane or hair bundles when doing so. Pull away the extracellular matrix with embedded otoconia by pulling with forceps; it behaves a bit like cotton candy. Try to just pull on the matrix, not damaging the sacculus.
6. Adjust your light to clearly see the sensory epithelium. Often a few otoconia will remain attached to the otolithic membrane allowing its visualization (Figure 4).
7. At this stage you can either use a protease treatment to loosen the otolithic membrane or you can manually remove it. The protease treatment is the most gentle way to do

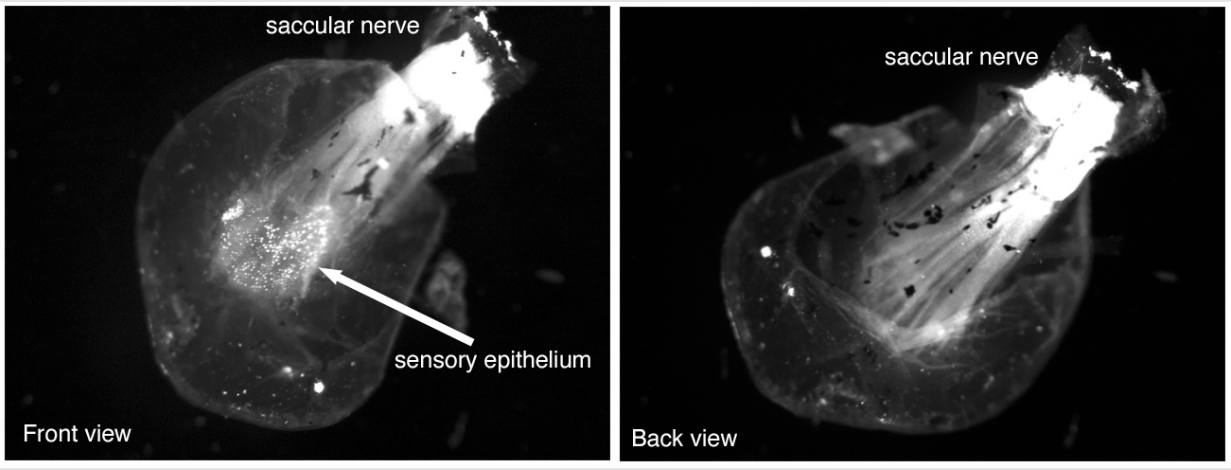


**Figure 4. Front view and back view of an isolate bullfrog saccular macula.** A few number of otoconia are still bound to the otolithic membrane making the kidney-bean shaped sensory epithelium evident.

this. For protease treatment, incubate the sacculus in 50 ug/ml of Type XXIV protease (Sigma) in frog standard saline for 20 min. at room temperature. Move the sacculus into a dish of standard saline after the treatment and manually remove the otolithic membrane.

8. To manually remove the otolithic membrane, use an eyelash glued to the end of a cotton applicator stick and tease up the edge of the otolithic membrane. Use your fine forceps to gently grab this edge and pull the otolithic membrane away. It is best to pull at about a 45 degree angle.

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**Solutions:**

10X Frog Standard Saline (1 Liter):

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| 110 mM NaCl | 64.28 grams |
| 2 mM KCl | 1.49 grams |
| 2 mM $MgCl_2$•6H2O | 4.07 grams |
| 4 mM $CaCl_2$•2H2O | 5.88 grams |
| 3 mM D-Glucose | 5.40 grams |
| 10 mM HEPES | 23.83 grams |

Dissolve in ~ 950 mLs of water. Titrate to pH 7.40 with NaOH. (When you dilute it to the 1x working concentration, the pH will drop to ~7.2) Bring volume up to 100% and double check the pH. Filter sterilize through a 0.2 um filter, and aliquot into 10-ml aliquots and freeze.