

CHAPTER 13

Histology for Finfish

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I. Introduction

Histology is an important field of fish health that can often detect subtle conditions or early signs of disease not easily recognized on gross examination. Changes at the cellular level and in the function of key organs are the first indications of mal-adjustment to stressors that may eventually lead to poor health or disease. Histology in the Wild Fish Health Survey supplements general observations and pathogen screening of wild fish populations. Special projects such as water quality and contaminant studies can be augmented with histology to provide better insight into the environmental and/or physiological demands presented to fish in their natural environment.

II. Preparation of Finfish Tissue - salmonids and non-salmonid species

NOTE: Only live or moribund fish will be suitable for processing. Tissues in dead fish autolyze very quickly and will mask antemortem changes. Do not collect and process dead fish. Keep fish alive as long as possible during transport to the site of necropsy. Do not over-ice fish such that fixed tissues freeze while in transit. Frozen tissues are worthless for histological examination. Animals are euthanized in a solution of MS-222, or other appropriate anesthetic.

A.. Tissues should be preserved in Davidson's fixative, or a non-formalin, alcohol-based fixative such as Prefer® or Safe-Fix®. Bouin's fixative can be used if decalcification is needed. The volume of fixative should be ten times the volume of tissue. This is important since less fixative may result in tissue autolysis and worthless samples. After 48 hours, the Davidson's may be poured off and replaced with 70% ethyl alcohol for transport and storage to prevent tissues from becoming too hard and brittle which occurs when left in acid fixatives for long periods. Tissues can be left in alcohol-based fixative for longer periods. Also, the fixative poured off may be saved and strained of tissue fragments and used one more time for other samples if necessary.

B. Fish less than 3 cm may be fixed whole by dropping into preservative.

NOTE: Important! Remove egg yolk from sac fry before fixation.

C. Fish 4 cm-10 cm should have the abdomen slit with a scalpel or scissors, the intestine detached at the vent, and the internal organs pulled out slightly for proper fixative penetration.

D. Larger fish (11 cm-20 cm) will require on-site excision of 0.5-cm sections of major tissues and internal organs as listed. Do not send whole fish.

E. Excise the head (from just behind the opercular opening) and dissect 0.5-cm samples of liver, air bladder, head and mesonephric kidney, spleen, GI tract (esophagus, stomach, pyloric caecae, anterior and posterior intestine with attached adipose tissue and pancreas), heart, gonads. Also, take a 0.5-cm square of musculature and attached skin intersected by the lateral line midway between the head and tail on the right side of the fish. Take a second 0.5-cm section of muscle and skin from the body wall covering the viscera from the right side of the fish.

- F. Organs and tissue samples from a single fish should be placed in tissue-processing cassettes, 4 to 5 tissue samples to one cassette. Sample thickness should not exceed 0.5cm.
- G. Label each cassette with the fish identification and case number using a soft lead pencil or black Secureline II marker. Other inks may not be permanent in solvents. Place cassettes and the fish head in a jar of fixative.
- H. Fish larger than 20 cm also require that 0.5-cm portions of each major organ be utilized (if larger than 0.5 cm) and the whole head will be eliminated from the sample unless a lesion is present or brain is to be examined. In this case the head may be cut in half longitudinally to include any lesion and tagged for identification. If the head is not kept, the first right gill arch is excised and fixed before discarding the head. Organs and tissues from a single fish are placed in tissue-processing cassettes as above or otherwise numbered.
- I. External and internal abnormalities must be noted on the Submission Form (Appendix B in Chapter 2), and the particular fish sample identified. Be sure and include tissues from a lesion area if there is one observed. The Submission Form will also contain the label information below and must accompany the samples in a separate Ziploc® bag.
- J. A label with fish species, size range and life stage, date of sample, location of sample, and contact person's name, address, and telephone number must be placed within each of the sample jars. Use a pencil with soft lead for labeling so that the writing remains legible.
- K. Do not mix samples of different fish species within the same jar of fixative. Each species requires a separate sample jar.
- L. If shipping collected material, place sample jars containing alcohol and tissues and the Ziploc® bag containing sample submission data into a suitable shipping package with adequate packing material to prevent breakage. Plastic jars or containers for fixative and samples work best. Be sure lids are on tight and do not leak.

NOTE: Any quantity of alcohols and formalin solutions are dangerous goods and need to be shipped in accordance with special packaging and shipping requirements. Check with Federal Express, or the commercial carrier used, for specific instructions on shipping dangerous goods.

1. Special Procedures - for fingerlings, fry, and sac fry:

Decalcify fingerlings and fry - Chelate sac fry with EDTA to soften any residual yolk sac.

Cassette and block label designation for finfish

Adults:

- A = gill arch (decalcify), thyroid - thyroid follicle present beneath gill arches
- B = liver, gallbladder, spleen
- C = heart, thymus (excised from beneath opercula where they join the head)
- D = kidney, head kidney, air bladder
- E = gut (esophagus, anterior intestine, posterior intestine, rectum), pyloric caeca, pancreas
- F = stomach (cardiac and pyloric)
- G = brain, eye
- H = gonads

Juvenile:

- A = halved head (thymus, thyroid, heart, eye, brain, gill, head kidney)
- B = liver, gallbladder, spleen, kidney, gonads, air bladder
- C = stomach, pyloric caeca, intestine, rectum, pancreas

NOTE: Proper sectioning of the brain and eye in smaller fish will require that the head be halved longitudinally after fixation using a very sharp razor blade. Both halves are laid face down in the cassette for embedment after decalcification.

III. Fixation and Decalcification

Finfish adults, juveniles, fingerlings, fry and sac fry are all fixed in Bouin's, buffered formalin, or Davidson's fixative that is usually prepared in 10-L quantities (as Bouin's deteriorates with age). Under usual circumstances soft tissues should be removed from Bouin's after 24-48 hr to reduce brittleness from acidity of the fixative. For larger whole fry it is advantageous to leave in fixative for up to 2-3 days since the acidity will decalcify bones, allowing for whole sectioning.

NOTE: Whole swimup fry do not need to be decalcified and become too brittle unless removed after 4-8 hours of fixation in Bouin's. The removal of picric acid from the tissues cannot be overemphasized. Several changes of 50% alcohol for 4-6 hours is recommended, along with constant agitation. Store samples in 70% alcohol.

Bouin's Fixative (10 L)

dH ₂ O	7.07 L
Formalin (37-40%) (CH ₂ O)	2.37 L
Glacial acetic acid (CH ₃ COOH)	476 ml
Picric acid (2,4,6-(NO ₂) ₃ C ₆ H ₂ OH)	71.0 g

Dissolve picric acid in water, then add other ingredients and leave on magnetic stirrer overnight. DO NOT HEAT!

Bouin's Fixative is also available from PolyScience(1-800-645-5825), catalog # S129.

10% Buffered Formalin

	<u>1 L</u>	<u>20 L</u>
37-40% formalin (CH ₂ O)	100 ml	2 L
Sodium phosphate (monobasic) NaH ₂ PO ₄ H ₂ O	4 g	80 g
Sodium phosphate (dibasic)(Na ₂ HPO ₄)	6 g	120 g
dH ₂ O	900 ml	18 L

Decalcification Solution

Solution A = Sodium citrate (Na ₃ C ₆ H ₅ O ₇ · 2H ₂ O)	50.0 g
dH ₂ O	250 ml
Solution B = Formic acid 88% (HCOOH)	125 ml
dH ₂ O	125 ml

1. Mix A & B in equal portions for use - (leave tissues in for 8-12 hours).
2. Wash in running tap water for 2-4 hours.
3. Place tissues in tissue processor for usual cycle.

Alternately, place in Cal-Ex® for 24 hours. Rinse in running water 3-4 hours and process as usual.

IV. Tissue Dehydration and Infiltration

- A. After the tissues are in cassettes, preserved with fixative and moved into 70% alcohol, they are dehydrated in the Histokinette tissue processor. The 12 baths in the processor should already contain the following solutions:

- #1 70% alcohol
- #2 85% alcohol
- #3 95% alcohol
- #4 95% alcohol
- #5 95% alcohol
- #6 100% alcohol
- #7 100% alcohol
- #8 100% alcohol
- #9 Hemo-sol
- #10 Hemo-sol
- #11 melted paraffin
- #12 melted paraffin

These solutions will evaporate over time and should be topped up as needed. The temperature for the paraffin baths should be set at 58-60°C. Do not exceed 62°C or polymerization will occur. This will produce hard blocks resulting in difficult or impossible sectioning.

- B. Place the cassettes into the processor basket and attach the basket to the processor. Line the basket up so that it will begin the cycle in the 70% alcohol bath #1.
- C. Program the dehydration cycle by setting the clock timer for 24 hrs at 12 two hr intervals. The processor will now advance the basket through the 12 baths at the two hour intervals when the cycle is activated. NOTE: Before starting the processor, think about the timing. The cycle will end in 24 hours. Make sure that you will have sufficient time to embed the tissues immediately following the completion of the cycle. An example would be to start the cycle at 1 PM on Monday for a completion time of 1 PM on Tuesday. This would leave enough time Tuesday morning to allow melting of the paraffin in the embedder and to embed two baskets of cassettes in the afternoon. Do not allow the tissues to remain in melted paraffin any longer than necessary. Excessive time in melted paraffin can cause tissues to become brittle and they become difficult to cut.

V. Embedding Tissues into Paraffin Blocks

- A. Turn on the paraffin bath in the embedder several hours prior to the time that the tissue processor cycle will end. You can also set the internal timer to automatically turn the embedder on at the appropriate time. This will allow the paraffin time to completely melt.
- B. Shortly before the scheduled embedding, turn on the cold plate so that it can become well chilled.
- C. When the tissue processor cycle ends, remove the basket from the final paraffin bath and pour the cassettes into the melted paraffin bath of the embedder.
- D. Now you are ready to begin embedding.
 - 1. Dispense enough paraffin into an embedding block mold to just cover the bottom. Place the mold on the hot plate of the embedder.
 - 2. Remove a cassette from the paraffin bath of the embedder and place it on the hot plate.
 - 3. Open the cassette and discard the lid. Using forceps, transfer the tissue sample(s) from the cassette to the mold.
 - 4. Place the mold on the cold plate. Using rounded forceps, gently press each tissue piece to the bottom of the mold. This must be done quickly. Allow the paraffin to set up just enough to hold the tissues in place. **DO NOT ALLOW THE PARAFFIN TO COMPLETELY HARDEN.**
 - 5. Quickly move the mold back to the hot plate and place the cassette bottom onto the mold like a cap.
 - 6. Fill the mold with melted paraffin from the dispenser of the embedder.
 - 7. Return the mold to the cold plate to cool.

- E. Once the block has completely solidified, it may be popped out of the mold and stored on the cold plate or in the freezer until you are ready to cut.

VI. Cutting Paraffin Blocks and Mounting Sections on Glass Slides

A. Preparation of materials

1. About an hour prior to cutting blocks, turn on the cold plate of the embedder and the water bath (start with fresh water each day). The water bath should be set at 45°C-48°C.
2. Sprinkle gelatin crystals (using a salt shaker) over the surface of the water bath. The gelatin is used to adhere the tissue section to the slide and helps provide a wrinkle-free bond between the tissue and the slide. If a heated stain is to be used, do not use gelatin. Instead use slides previously dipped in 5% Elmer's® glue solution.

Recipe for Elmer's® glue dipped slides (for use with heated stains):

- a. Make 5% glue solution with distilled water (from commercial bottle of Elmer's® glue).
 - b. Heat in microwave.
 - c. Allow air bubbles to disperse.
 - d. Dip slides and allow to air dry.
3. Store glass slides from the box in 100% alcohol and wipe clean as needed. Using clean glass slides can not be overemphasized.
 4. Place blocks to be cut on the cold plate.
 5. Check the blade in the microtome and replace if nicked or scratched. Use disposable blades, they are always sharp and clean. Because disposable blades are at a fixed angle, there is little tissue loss when refacing a block if recutting is required.

B. Cutting sections

1. Clamp a block securely into the microtome chuck and begin cutting. Ideal sections will be between 2-6 microns thick.
2. Once you are able to cut a flat ribbon of whole sections, transfer the ribbon to the water bath, taking care to gently stretch out the wrinkles as the ribbon makes contact with the warm water surface.
3. Tissue's fixed in Bouin's tend to fragment during cutting (due to the picric acid). If you are having difficulty obtaining good sections, try placing a gauze pad that has been soaked in 2% ammonia on the face of the block for a few minutes. Then recut. If fragmentation or compression still occurs, place the block and the ammonia soaked gauze in a sealed bag and leave overnight. Recut.

4. Separate the desired sections from the ribbon by gently pulling the ribbon apart using two small, fine bristled paint brushes. Stubborn sections may be separated with a dissecting needle dipped in Hemo-Sol® or a tool heated in an open flame which will “cut “ the sections apart.

C. Mounting sections

1. Submerge a clean glass slide into the water bath under the desired sections. Gently pull the slide out of the water at an angle holding the sections in place on the slide with the paint brush.
2. Lean the slide upright to drip dry for a few moments. Heat fix the slide by placing it on the slide warming area of the water bath (or any slide warming tray) for a second or two. Caution! You do not wish to melt the paraffin in the section, just warm the slide.
3. Label the slide with the accession number, block number, slide number, date and your initials.
4. Place the slides in a staining rack. Typically, sections for three slides are cut from each block. Two of these slides are stored in a rack for standard H&E staining at a later date while the third slide is stored in a second rack and will not be stained with the other two. Instead, this third slide is used as a back-up should there be a staining problem or a need for a special stain.
5. All the slides now in racks should be dried overnight at 40-50°C prior to staining. This step helps to prevent wash-offs during the staining process. If the paraffin in the sections melts, the temperature is too high and tissue artifact will occur.

VII. Routine Staining of Paraffin Sections - Hematoxylin and Eosin

A. Hematoxylin Solution (Harris Formula)

Purchase already prepared; it is inexpensive and gives reproducible results (500 ml is less than \$10). It contains no mercury and is available in both acidified and unacidified formulations.

Eosin Y

1% stock solution

Eosin Y, water soluble	1 g
dH2O	20 ml

Dissolve and add:

Alcohol 95%	80 ml
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Working Solution

Eosin stock solution	1 part
Alcohol 80%	4 parts

Just prior to use add 0.5 ml of glacial acetic acid to each 100 ml of Hematoxylin and the working Eosin solution.

B. General H & E staining

The basic procedure includes getting rid of the paraffin in the sections (deparaffinization) and rehydration of the tissue so that the H & E stains may be used. This is followed by dehydration again so that the stained section may be mounted in a permanent medium under a glass coverslip.

Standard H&E schedule

- #1 Xylene - 10 minutes or Histo-Clear® - 15 minutes (at least). Change the Histo-Clear® after several racks (check slides).
- #2 Xylene - 10 minutes or Histo-Clear® - 15 minutes (at least)
- #3 100% alcohol - 1 minute
- #4 100% alcohol - 1 minute
- #5 95% alcohol - 1 minute
- #6 95% alcohol - 1 minute
- #7 tap H₂O - 10 minutes
- #8 Hematoxylin - 10 minutes (varies with section thickness and animal species)
- #9 tap H₂O - 4 dips
- #10 Acid alcohol - 3 - 10 dips
- #11 Tap H₂O - 4 dips
- #12 Ammonia water - 3 - 5 dips
- #13 Tap H₂O - 20 minutes
- #14 Eosin - 2 seconds to 2 minutes (varies with section thickness and animal species)
- #15 95% alcohol - 2 minutes
- #16 95% alcohol - 2 minutes
- #17 100% alcohol - 3 minutes
- #18 100% alcohol - 3 minutes
- #19 Xylene or Histo-Clear® - 2 minutes
- #20 Xylene or Histo-Clear® - 2 minutes

Staining times will vary with thickness of sections, age of stain, and animal species. Thinner sections will require increased staining times. This staining schedule is based on sections of fish tissues 2-3 μ thick.

VIII. References

Luna, G. Lee. Editor. 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd Edition. McGraw-Hill Book Company, New York.

Thompson, S. W. 1966. Selected histochemical and histopathological methods. Charles C. Thomas, Publisher, Illinois.