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 National 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.

This particular chapter of the manual is unique in that for the purposes of the National Wild Fish Survey histologic examination of the tissues is ancillary, and thus up to the discretion of the Fish Health Biologist. In addition, the variation in equipment among the labs (especially tissue processors) and individual histologists' preferences make a specific standardized approach impossible. Instead, this chapter is intended to be a general guideline and offer tips.

II. Acknowledgements

The instruction and comments by John Morrison, Beth MacConnell and Sandy Pidgeon were invaluable for this chapter.

III. Preparation of Finfish Tissue

NOTE: Only live or moribund fish will be suitable for processing. Do not collect and process dead fish. Tissues in dead fish autolyze quickly and will mask ante-mortem changes. Keep fish alive as long as possible during transport to the site of necropsy. Animals are euthanized in a solution of MS-222, preferably buffered (with baking soda) to pH of water the fish came from, or other appropriate anesthetic.

Do not over-ice fish such that fixed tissues freeze while in transit. Frozen tissues result in artifacts that make interpretation of the results difficult.

A. Tissues should be preserved in Davidson's fixative, 10% neutral buffered formalin, or non-formalin, alcohol-based fixative such as Prefer® or Safe-Fix®. Davidson's contains acetic acid in it which results in some decalcification. Place the tissues into the fixative immediately after euthanasia. Handle the tissues carefully with forceps as excessive pressure can cause cellular damage which is visible microscopically in the tissue sections. The volume of fixative should be ten times the volume of tissue. This is important since less fixative may result in tissue autolysis, compromising the evaluation of the tissues. For most tissues, after 24-48 hours, the Davidson's should 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. This transfer should be done in a fume hood. Tissues can be left in alcohol-based fixative for longer periods.

B. In order to get the fish tissues in to fixative as quickly and with as little tissue damage as possible, you may want to pre-label your containers or cassettes. When labeling cassettes with the fish identification/case number, use a soft lead pencil or a marker specifically designed to withstand the solvents used in tissue processing. When handling the tissues, use tissue-saving instruments (i.e. not rat-toothed forceps) and minimize pulling on organs. Work quickly so that the tissues do not dry out.

C. Fish less than 3 cm may be fixed whole by dropping into preservative. Slightly larger fish can still be fixed whole if the caudal peduncles are removed.

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

D. 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.

E. Larger fish (>11 cm) will require on-site excision of 0.3-cm sections of major tissues and internal organs as listed. Do not send whole fish. Depending on the situation in the field, the collector may elect to do the "cutting in" (preparing the samples for placing in cassettes) or perform the gross dissection in the field, to later be "cut in" at the lab. In any case, the tissues should be no thicker than 0.3 cm in at least one dimension, allowing for adequate penetration of fixative into the organ. Exceptions to this rule would include heads and brains, among others. When cutting in organs that have a capsule, try to include some of the capsule for orientation when embedding and reading the slides.

F. At the discretion of the fish health biologist, the number and type of tissues collected may vary. If the sampler is unsure as to how many tissues to take, more is better as tissues can be discarded later if they are not necessary. Using the necropsy procedures outlined in Chapter 4, open the body cavity of fish (if histo preparations only, sterility is not essential). Where possible, try to get samples approximately 0.5-cm x 0.5 cm X 0.3 cm of liver, head and mesonephric kidney, spleen, GI tract (esophagus, stomach, pyloric caecae, anterior and posterior intestine with attached adipose tissue and pancreas), heart, gonads. Use a sharp scalpel or razor blade to cut organs. Using scissors, cut the top and bottom of the gill arch(s). Carefully remove one or more gills, taking care to only handle them at the cut edge. Using the scalpel blade, cut a 0.5 x 0.5 x 0.3-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. Excise the head (from just behind the opercular opening). If brain in desired, shave off the skull just on the dorsal aspect of the brain, taking care not to disturb the brain. If the entire head is desired, For examination of the head for fingerling size fish or smaller, cut the head sagittally off center, to allow for adequate penetration of the fixative. Refer to the AFS Bluebook for diagrams, if needed.

G. Organs and tissue samples from a single fish should be placed in tissue-processing cassettes, up to 4 to 5 tissue samples to one cassette (depending on size). If tissues are being compacted by the cassette or lid, then reduce the number and/or size of the tissues in the cassette. In general, multiple tissues, especially of unlike textures, will make cutting

the blocks more difficult. Try to group tissues of like textures in the same cassette. Gills should be in cassettes of their own (unless whole fish or heads) to facilitate cutting.

H. 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. Lesions should include normal and abnormal tissue. The Submission Form will also contain the label information below and should accompany the samples in a separate sampling bag.

I. Do not mix samples of different fish species within the same jar of fixative. Each species requires a separate sample jar. Individual fish can be kept separate by cassette or individual jars.

J. If shipping collected material, place sample jars containing alcohol and tissues and the high quality sample 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. Wrap electrical tape around the lid seal several times. Make sure everything is double bagged with a high quality sampling bag.

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.

l. Special Procedures - for fingerlings, fry, and sac fry:
Decalcify fingerlings and fry – If the yolk sac is not removed, chelate sac fry with EDTA to soften the yolk sac.

2. Larger fish heads may need to fix for up to 72 hrs.

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 embedding after decalcification.

IV. Fixation and Decalcification

Finfish adults, juveniles, fingerlings, fry and sac fry are can be fixed in buffered formalin, or Davidson's fixative that is usually prepared in 10-L quantities. Soft tissues should be removed from Davidsons 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 48-72 hr since the acidity will decalcify bones, allowing for whole sectioning.

NOTE: Whole swim up fry do not need to be decalcified and become too brittle unless removed after 4-8 hours of fixation. Store samples in 70% alcohol.

Davidson's Fixative

95% Ethanol	600 mL
Acetic acid	200 mL
Formalin	400 mL
Deionized water	600 mL

10% Buffered Formalin

	1 L	20 L	
Formalin	100 mL		2 L
Sodium phosphate (monobasic) $NaH_2PO_4H_2O$	4 g	80 g	
Sodium phosphate (dibasic)(Na_2HPO_4)	6 g	120 g	
dH_2O	900 mL		18 L

Decalcification Solution

Solution A = Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	50.0 g
dH_2O	250 mL
Solution B = Formic acid 88% (HCOOH)	125 mL
dH_2O	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.

NOTE: Always wear gloves when handling fixatives, especially Bouins, 10% NBF, and Davidson's, all contain cancer causing chemicals.

V. Tissue Dehydration and Infiltration

A. After the tissues are in cassettes, preserved with fixative and moved into 65%- 70% ethanol, they are dehydrated in a tissue processor. Isopropyl alcohol may be used if ethanol is not available, but may compromise tissue integrity. The solutions and times can be processor dependent, refer to the manufacture's instructions of your equipment. The following is a general procedure for processing.

#1 70% alcohol
#2 70% alcohol
#3 95% alcohol
#4 95% alcohol
#5 95% alcohol
#6 100% alcohol
#7 100% alcohol
#8 100% alcohol
#9 Pro-Par®or other comparable clearant
#10 Pro-Par® or other comparable clearant

#11 Paraplast extra ® or other comparable melted paraffin
#12 Paraplast extra® or other comparable melted paraffin

B. These solutions will evaporate over time and should be topped up as needed and replaced after processing a set number of cassettes or a specified time period (see manufactures' instructions) The temperature for the paraffin baths should be set at 1 - 2º C, no higher than 3-4°C above the melting point of the paraffin you are using. (59-60°C for Paraplast extra®. Do not exceed 62°C or polymerization will occur. This will produce hard blocks resulting in difficult or impossible sectioning.)

C. The exact program times may be dependent on the type of equipment used at each lab. Do not allow the tissues to remain in melted paraffin any longer than necessary. Excessive time or heat in melted paraffin can cause tissues to become brittle and they become difficult to cut.

VI. Embedding Tissues into Paraffin Blocks

A. Turn on the paraffin bath in the embedder prior to the end of the processing cycle in sufficient time to allow the paraffin to completely melt (may be several hours). (You may be able to set the internal timer to automatically turn the embedder on at the appropriate time.)

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 put the cassettes into the melted paraffin bath or the cassette chamber of the embedder. It is important to start embedding ASAP to prevent the tissues from becoming brittle. Try to embed all of your blocks within at least 2 hours of the processor finishing.

D. Place the cassettes flat on the bottom of the holding chamber to keep the tissues warm until they are embedded with wax. Do not leave them in the chamber too long without wax as they will become brittle.

E. During Embedding:

1. Dispense enough paraffin into an embedding block mold to 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, gently transfer the tissue sample(s) from the cassette to the mold.
4. Keeping in mind the orientation of the block on the microtome, try not to place hard tissues (boney or containing grit or scales) above or below other tissues.

This placement will make cutting easier. (Ideally, use separate cassettes for hard tissues).

5. Keep the tissues away from all the edges of the mold. Having plenty of wax around the tissues allows for a better ribbon when cutting.
6. Consult your histologist on how the tissues should be oriented (i.e. the hollow viscera can be oriented in cross-section, sagittally or longitudinally).
7. Place the mold on the cold plate. Using rounded-tipped 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.
8. Quickly move the mold back to the hot plate and place the cassette bottom onto the mold like a cap.
9. Fill the mold tot the top of the mold with melted paraffin from the dispenser of the embedder (paraffin will shrink as it hardens). If the bottom layer of wax is allowed to cool too much, the block will likely split when cutting.
10. Return the mold to the cold plate to cool.

F. Once the block has completely solidified, it may be popped out of the mold. If the cassette cannot be easily popped out, do not force it. The block may break, if it does, then it can still be re-embedded. Note: re-embedding increases the tissue brittleness and should be avoided if possible. If the blocks are difficult to remove, try cooling the blocks down more, or go to a commercially available mold release spray prior embedding. Once the block is removed, use a scraper to remove any excess wax along the edges of the block. If this wax is not removed than it may be difficult to hold the block in the chuck of the microtome in a consistent manner.

G. At this point, if blocks are going to be cut that day they can be stored in the freezer until you are ready to cut. For longer term storage, store in a cool, dry place where the temperature will not exceed the melting point of the wax.

VII. Cutting Paraffin Blocks and Mounting Sections on Glass Slides

A. Preparation of materials

1. The blocks should be cold prior to starting. If they are not cold from the embedding center, they can be placed in the refrigerator prior to trimming. The water bath should be set at 45°C-48°C (below melting point of wax). If air bubbles in water bath are a problem, use a paint or make-up brush to remove the bubbles prior to cutting sections. In areas where there are excessive minerals or chlorine in the tap water, consider using de-ionized water.
2. Standard Glass slides can be used or if tissues do not remain attached to the slides during the staining process, electrostatic (Plus) or Poly-Lysine treated slides may be used. Pre-labeling maybe helpful.
3. Place blocks to be trimmed on the cold plate or ice block.

4. Check the blade in the microtome and replace if nicked or scratched. Use disposable blades, they are always sharp and clean. There is little tissue loss when refacing a block if recutting is required if blades are held at a fixed angle.

B. Cutting sections

1. Clamp a block securely into the microtome chuck and begin cutting. Ideal sections will be between 2-6 microns thick. Try to be consistent on which way they block label is placed in the chuck (i.e. always up or always to the right). This consistency will be helpful when you go to re-cut blocks…less re-facing waste.
2. You may choose to "face off" (trim off the wax until you have exposed the tissues to be cut and the blade comes in contact with the entire block) all the blocks to be cut for the day prior to actually cutting any sections. This allows you to use one blade for all the trimming. In addition, it gives time for your tissues to become re-hydrated on the block of ice with water. Once the blocks have been faced off, add a small amount of water to your ice block to allow rehydration of the tissues. Rehydration will improve tissue sectioning and ribbon integrity. The tissues require varying amounts of time for re-hydration, but 20-30 minutes for most tissues is adequate. If you decide to do this, keeping the block label in the same place in the chuck and removing all the excess wax around the block is even more important. Use a fresh blade when starting to section your blocks.
3. After the tissues are re-hydrated, you are ready to cut your sections. Once you are able to cut ribbon of whole sections, transfer the ribbon to the water bath. Possible ways of making the transfer include two small paint brushes, coverslipping forceps, and fingers. If you are using fingers, consider wearing gloves to prevent your epithelial cells from ending up on your sections. Regardless of the technique, try to gently stretch out the wrinkles as the ribbon makes contact with the warm water surface. If a bubbles occur in the sections: 1) reduce heat 1) gently dab the bubble with a paintbrush to remove.
4. Separate the desired sections from the ribbon by gently pulling the ribbon apart using two small, fine bristled paint brushes. Sections may also be separated with tool (knife) heated in an open flame or heating block which will "cut "the sections apart. Do not put the heated tool too close to the tissues. You can also eliminate the need for separating the sections in the bath by dipping the slide under the ribbon and wiping the excess ribbon off, after allowing the slide to dry.

C. Mounting sections

1. Submerge a clean, labeled glass slide into the water bath under the desired sections. Gently pull the slide out of the water at a slight angle. Consider cutting at least two sections per block to allow for H&E staining and one additional stain if needed.
2. Lean the slide upright to dry. Use racks designed to hold the slides at an angle to facilitate drying with no water underneath the sections.
3. Once the slides are dry. Place the slides in rack (which may be a staining rack)to be placed in oven.

4. All the slides now in racks should be dried for 30-40 minutes, up to an hour at 40-50°C prior to staining. Note: Heat is detrimental to the tissues. 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. Alternatively, you can allow the slides to dry overnight prior to staining.

VIII. Routine Staining of Paraffin Sections - Hematoxylin and Eosin

There are many H&E stains and protocols that are readily available either as components or part of a kit. Here is an example of one that has worked well for fish tissues:

A. Hematoxylin Solution (Harris Formula) and Eosin Y.
Purchase already prepared; it is inexpensive and gives reproducible results. Ordering information is listed at the end of this chapter.

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	Clearant -(i.e. Propar®)	5 minutes in each container
#2	Clearant - (i.e. Propar®)	5 minutes
#3	Clearant – (i.e. Propar®)	5 minutes
#4	100% alcohol	5 minutes
#5	95% alcohol	5 minutes in each container
#6	95% alcohol	5 minutes
#7	65-70% Alcohol	5 minutes
#8	deionized water	30 seconds
#9	Hematoxylin	4 minutes
#10	Running water	10 minutes
#11	Acid Alcohol	30-45 seconds (approx. 15 dips)
#12	Tap Water	Rinse
#13	Scott's	1 min.
#14	60-75% Alcohol	Quick Rinse
#15	Eosin	1.5 minutes
#16	65-70% alcohol	Quick Rinse
#17	95% alcohol	Quick Rinse in each container
#18	95% alcohol	Quick Rinse
#19	100% alcohol	3 minutes in each container
#20	100% alcohol	3 minutes
#21	100% alcohol	3 minutes
#22	Clearant –(Propar®)	3 minutes in each container
#23	Clearant –(Propar®)	3 minutes
#24	Clearant –(Propar®)	3 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 5 µ thick.

Recipes:

Acid Alcohol: 7 mL Acetic acid
252 mL 65-75% ethanol
* mix in staining container *

Scotts: 189 mL distilled water
29 mL of Scott's Stock
* mix in staining container *

Scotts Stock: 1 gm Potassium bicarbonate
10 gm Magnesium sulfate
500 mL distilled water
* Mix potassium bicarbonate and magnesium sulphate into 500 mL of distilled water. Store stock Scott's in refrigerator.

B. Special staining
Examples of special stains include but are not limited to Giemsa, Steiner, Silver, Periodic Acid Schiff, Methylene Blue, and Gram Stains. Protocols for these stains can be obtained in one of the histology texts listed on the reference page.

IX. Cover slipping

A. Try to coverslip in a hood, even the xylene substitutes are not "non-toxic"

B. There are many comparable ways to coverslip, one method is outlined below:

1. Put a clean paper towel down.
2. Have slides, appropriate sized coverslips (No. 1 thickness), and mounting media available. There are many different mounting medias, consider trying a low viscosity media compatible with the xylene substitutes since the media is not as soluble in Propar® or other xylene substitutes as it is in xylene.
3. Keep the slides waiting to be coverslipped in clearant.
4. Wipe the back of the slide to be coverslipped with a paper towel. Place the slide on the paper towel.
5. Put the coverslip down directly in front of the slide and centered on the sample. The coverslip should be flat on the paper towel.
6. Put a few drops of mounting media on the center or the slide or in a line, depending on the size of the coverslip.

7. Pull the back corners of the slide upward and over the coverslip until the side is face down on the coverslip. This method will pull the mounting media over the slide by using low angle capillary action. Placing the coverslip flat on the slide will induce air bubbles.
8. If the mounting media is too thick, gently press on the coverslip with a pencil eraser, or put the slide face down on the paper towel and gently press over then entire surface of the slide.
9. Air bubbles can also be removed with a pencil eraser. Check for air bubbles 30 minutes after coverslipping.

X. Reagent Source List

A.	**ExCell plus**	**FXEXPCS**	**American Master Tech**
B.	**Paraplast extra**	**15159-486**	**VWR**
C.	**Propar**	**511**	**Anatech**
D.	**Bouins**	**16045-8**	**PolySciences**
E.	**Permount**	**SP15-100**	**Fisher**
F.	**Harris Hematoxylin**	**HXHHELT**	**American Master Tech**
G.	**Harris Hematoxylin**	**842**	**Anatech**
H.	**Eosin Y**	**STEOSLT**	**American Master Tech**
I.	**Eosin Y**	**832**	**Anatech**
J.	**Giemsa Powder**	**11700**	**Sigma**
K.	**Jenners Dye**	**861197**	**Sigma**

Manufactures websites (this is not an exhaustive list)

www.mastertechs.com	**American Master Tech**
www.polysciences.com	**Polysciences**
www.fisher.com	**Fisher**
www.vwr.com	**VWR**
www.sigmaaldrich.com	**Sigma**
www.rallansci.com	**Richard Allan**
www.sakura.com	**Sakura**
www.anatechltdusa.com	**Anatech**

XI. Maintenance of Equipment

The amount and type of maintenance required will be depend on the specific piece of equipment in each lab (refer to manufacture's instructions) and the volume of tissues processed. However, there should be some schedule for changing of reagents and wax as physical/chemical changes can occur even with light usage.

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