MOLLUSCS PROCESSING FOR DIAGNOSIS BY HISTOLOGY

SUMMARY

Editions

Edition	Date	Updated part
# 1	26/09/2006	Creation
# 2	08/10/2006	Modification of § 2 & 6.6
# 3	26/05/2011	Creation of § 6.4, modification of § 5.1, 6.5 and 7

MOLLUSCS PROCESSING FOR DIAGNOSIS BY HISTOLOGY

1. Scope

This procedure explains the techniques used for histological processing of common bivalves and abalones. It explains the processes of dissection, fixing, embedding and cutting tissue with a microtome before staining slides for histological examination.

2. References

- OIE. Manual of Diagnostic Tests for Aquatic Animals, current edition, Paris, France.
- Howard D.H., Lewis J.L., Keller B.J. & Smith C.S. (2004). Histological Techniques for Marine Bivalve Mollusks and Crustaceans, NOAA Technical Memorandum NOS NCCOS 5, 218 p.

3. General information

Tissue fixation preserves cellular details for examination by microscopy. An ideal fixative quickly penetrates tissue to prevent post-mortem damaging. It coagulates cell proteins by binding them together and hardens tissue to allow further histological processing (dehydration, embedding in paraffin and cutting with a microtome) without changing too much the shape of each organ. Embedding is the process of placing tissue in a firm medium to keep it intact when cutting sections with a microtome for histological examination

4. Equipment and environment

4.1. Equipment

- Scalpel or knife
- Razor blades (or used microtome blades)
- Gloves
- Tweezers
- Paper towelling
- Cassettes for histology
- Measuring cylinders
- Pots for tissue fixation
- Oven (42°C)to dry slides
- Histological slides
- Racks for histological slides
- Automatic tissue processor
- Embedding centre
- Cooling unit
- Metallic molds
- Microtome
- Heated waterbath
- Needle or paintbrush

4.2. Environment

- Well ventilated laboratory
- Fume hood

5. Preparation of fixatives

5.1. Reagents

- Ethanol 100%
- Ethanol 95%
- Ethanol 70%
- Xylene
- Paraffin
- Formaldehyde 36-40 %
- Filtered sea water
- Glycerin
- Acetic acid 99-100%
- Sodium dihydrogenophosphate (NaH_2PO_4, $2H_2O$)
- Distilled water
- Sodium hydroxyde pellets (NaOH)

5.2. Formulas for histology fixatives

Fixatives must be prepared under a fume hood.

Davidson's fixative can be used in routine survey. Formalin 10% in sea water is a general fixative, easily made and particularly interesting in the field or if travelling because of its simplicity.

Carson's fixative can be used for histology and allows also subsequent post-fixation with glutaraldehyde and osmium tetroxyde if transmission electronic microscopy (TEM) is needed for further investigation.

5.2.1. Davidson's fixative

Stock solution:

- Filtered sea water…….…….. 1200 ml
- Ethanol 95 % …….……. 1200 ml
- Formaldehyde 36-40 % …….. 800 ml
- Glycerin ….…… 400 ml

Working solution:

- Stock solution …….9 parts
- Glacial acetic acid……. 1 part (*add extemporaneously i.e. just prior to utilisation*)

5.2.2. Formalin 10% fixative

- Filtered sea water…….…….. 900ml
- Formaldehyde 36-40% …… 100ml
  pH neutral

5.2.3. Carson's fixative

- Dissolve in 900 ml of distilled water:
- Sodium dihydrogenophosphate ….....… 23.8 g
- Sodium hydroxide …….....… 5.2 g
- Then add:
- Formalin 36-40% ……..... 100 ml
  Mix thoroughly

6. Procedure

6.1. Preparing molluscs for histology

Open molluscs and quickly cut the adductor muscle(s) as close to the shell as possible (see the SOP "Opening bivalves"). Look for any clinical signs that can be observed on the shell (blister, boring sponge, brown ring, malformation, mud worm tunnel, pearl, pustules, scar) or on the soft parts (abscess, abnormal pigmentation, gill erosion, pustule, watery condition). Gently remove the body from the shell and put it on a paper towelling prior to slicing. Parts of abnormal tissue can be cut and fixed separately, for example in glutaraldehyde for Transmission Electron Microscopy (TEM).

6.2. Slicing molluscs before histology process

6.2.1. General information

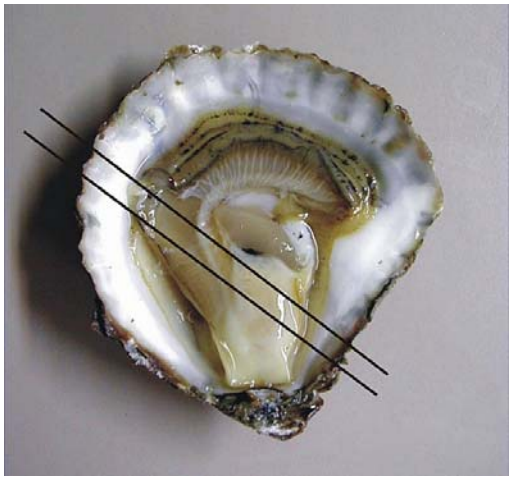
Many molluscs from the same family share the same organisation. The general slicing process for the families of oysters, mussels, clams (or cockles), scallops and abalones which represent most of the molluscs produced in Europe is described. Very small molluscs (up to 2 cm long) can be fixed entirely.
Each proposed slicing plan is made to include most of the organs like digestive gland, gonad, intestine, gills, kidney.

6.2.2. Oysters

Slice must be made as following:

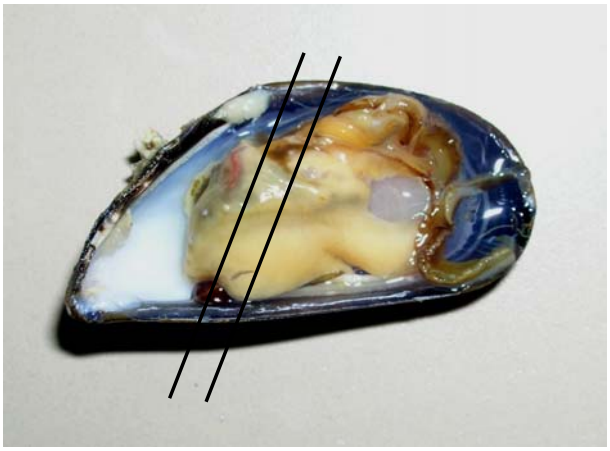
Spat (*sagittal plane*)

Adult



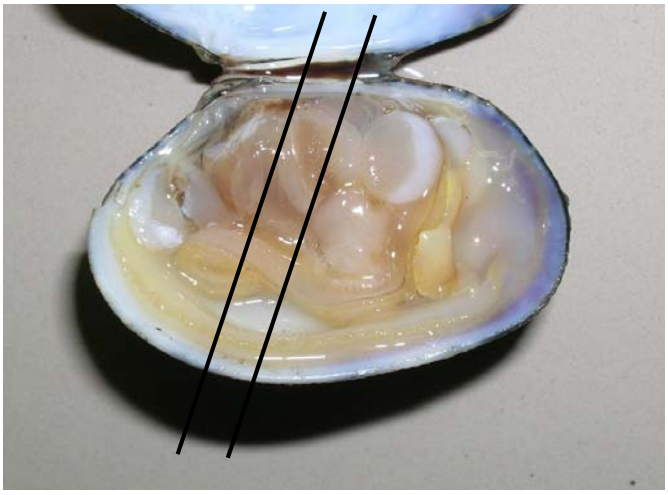
6.2.3. Mussels

Slice must be made as following (for **spat**, cut mussels by the saggital plane):



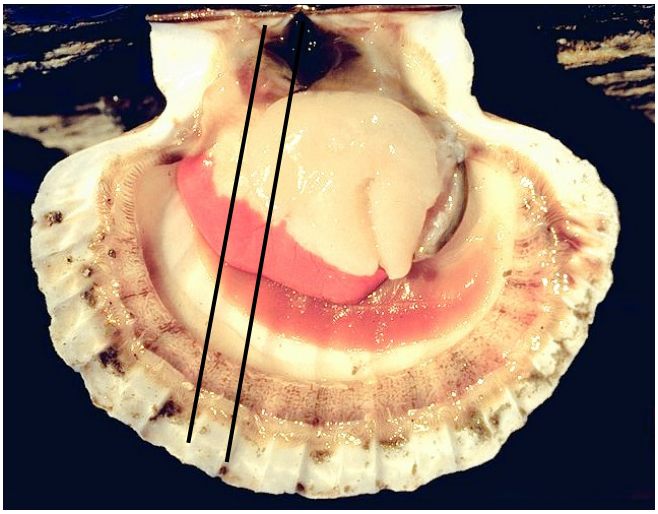
6.2.4. Clams

Slice must be made as following (for **spat**, cut clams by the saggital plane):



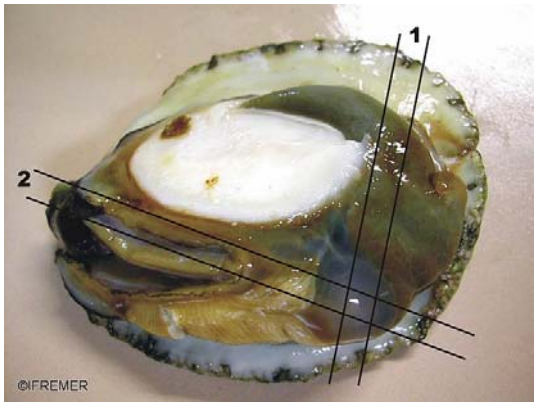
6.2.5. Scallops

Slice must be made as following (young and adult):



6.2.6. Abalones

2 slices must be made as following:



6.3. Fixation

Tissue fixation preserves cellular detail for examination by microscopy. Many fixatives are used and each one has its own properties for preserving such or such parts of the cell. OIE recommends the use of Davidson's fixative for general molluscan pathology. If *in situ* hybridation (ISH) is planned after histology (for confirmatory diagnosis for example) do not let the fixative process exceed 48 hours for better results; otherwise acetic acid would interfere with DNA preservation. Another possibility is to use Davidson's fixative without the acetic acid (i.e. use only the stock solution). Other fixatives can be used (see Howard et al., 2004, for more information).
Put slices of tissue of no more than 5 mm thickness in cassettes. Cassettes must be identified with the code of the sample and the number of the individual. Try to carefully set the tissue in the cassette so that each cut organ can be visible. If needed you can put other parts of the body in the cassette. Work must be done under a fume hood.

1. Put the slice of tissue in the cassette and orient it carefully
2. Immerge the cassette in the fixative (around 10 volumes of fixative for 1 volume of tissue)
3. Fixation should last 24 h minimum

6.4. Storage

If you need to keep fixed tissue for several days or weeks before further processing, transfer fixed tissue into **70% alcohol**.

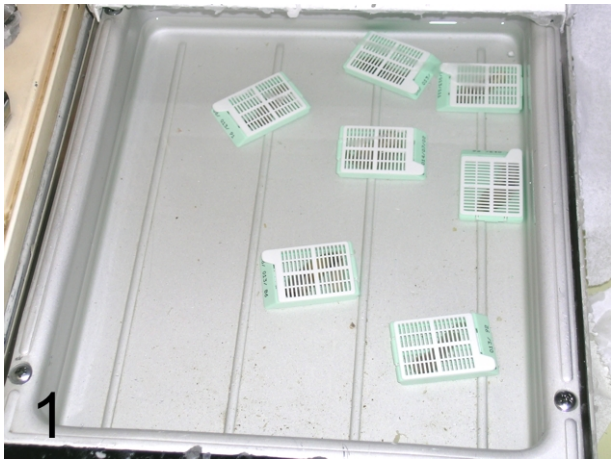
6.5. Tissue dehydration and infiltration

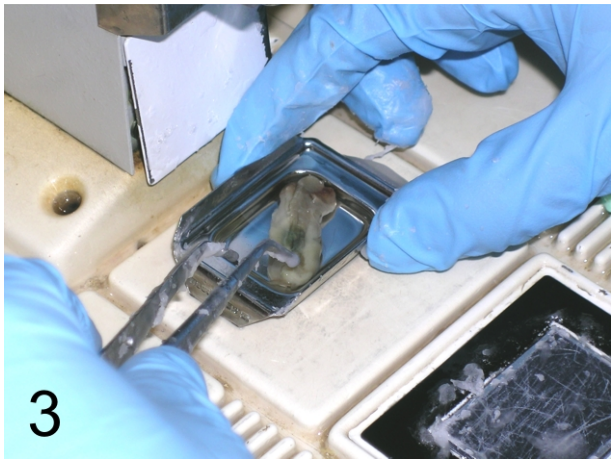
Once samples are fixed, they must be dehydrated and infiltrated with paraffin. This can be done manually or automatically by using an automatic tissue processor.
Here is an example of dehydration and infiltration program (process time can vary with the thickness and size of tissue):

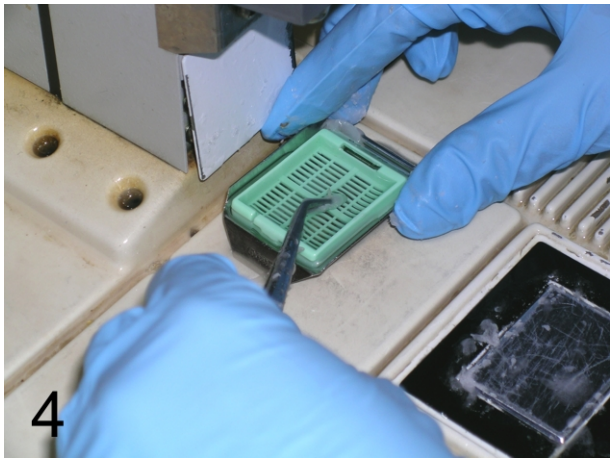
1. Ethanol 70% (30 minutes)
2. Ethanol 95 % (30 minutes)
3. Ethanol 95 % (30 minutes)
4. Ethanol 100 % (15 minutes)
5. Ethanol 100 % (30 minutes)
6. Ethanol 100 % (60 minutes)
7. Xylene (30 minutes)
8. Xylene (60 minutes)
9. Xylene (60 minutes)
10. Paraffin (45 minutes)
11. Paraffin (45 minutes)
12. Paraffin (45 minutes)
13. Paraffin (waiting bath before embedding)

6.6. Embedding

Embedding is the process of placing tissue in a block of paraffin to allow cutting sections with a microtome. Embedding centres are available with built-in paraffin baths and cooling units.
Remove tissue cassettes from the tissue processor and put them in the heated paraffin bath (picture 1). Take one cassette, place it on the heated surface and open it (picture 2). Put some paraffin in a mould (1/4 maximum). Take tissue from the cassette with heated forceps and orient it in the mould without trapping air bubbles (picture 3). Put the cassette on top of tissue (picture 4) and fill the mould with heated paraffin. Place the mould onto the cooling unit of the embedding centre. When the paraffin block has cooled, remove it from the mould for trimming and sectioning.







6.7. Sectioning

Good sectioning requires training and experience for the technician as well as a properly prepared material (i.e. well fixed and preserved and well dehydrated and embedded tissue). It is recommended that paraffin blocks be rough cut at room temperature and then precooled at 4 to 5°C (stored overnight in a fridge for example) before sectioning.

1. Rough cut blocks (using old blades for example)
2. Precool the paraffin blocks in a fridge or on a cooling table
3. Trim block until tissue is fully exposed
4. Set the microtome to 2-3 µm for section thickness
5. Cut ribbon of paraffin sections with the microtome
6. Put the ribbon on the heated waterbath (change water everyday)
7. Separate the sections
8. Dip coded slides under the tissue section and raised the slide from the water (guiding the section with a needle or brush)
9. Place the slides vertically in a rack to drain excess water
10. Dry in an oven or at room temperature
    Slides can be stored vertically on a rack or in a staining holder before staining process.

7. Safety information

Many hazardous chemicals are used during the histological process. All of them come in containers with special labels identifying their hazard characteristics such as **flammable**, **corrosive**, **reactive**, **toxic**, etc. Information on MSDS (material safety data sheets) can be found on Internet (for example: http://www.chemexper.com/). The **flash point** of a flammable product is the lowest temperature at which it can form an ignitable mix with air. Note that some paraffin media contain DMSO (dimethylsulfoxyd) which is slightly toxic: use of protective gloves is recommended.

Absolute Ethanol *(use under a fume hood)*

Eye: Causes severe eye irritation.
Skin: Causes moderate skin irritation.
Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhea.
Inhalation: Vapours may cause dizziness or suffocation.

Flash point: 16.6°C

H225 - Highly flammable liquid and vapor

Xylene *(use under a fume hood)*

Eye: Causes severe eye irritation .
Skin: Exposure may cause irritation. Prolonged contact may cause dermatitis.
Ingestion: May cause central nervous system depression, kidney damage and liver damage.
Inhalation: High concentrations may cause central nervous system effects characterised by nausea, headache, dizziness, unconsciousness and coma. Vapours may cause respiratory tract irritation. Irritation may lead to chemical pneumonitis and pulmonary oedema.

Flash point: 32°C

H332 - Harmful if inhaled
H315 - Causes skin irritation
H312 - Harmful in contact with skin
H226 - Flammable liquid and vapor

Formaldehyde *(use under a fume hood)*

Eye: Causes irritation. May result in cornea injury.
Skin: Causes skin irritation. Harmful if absorbed through the skin.
Ingestion: Causes gastrointestinal irritation with nausea, vomiting and diarrhea. May be harmful if swallowed.
Inhalation: Harmful if inhaled. Causes respiratory tract irritation.
Mutagenic effects have occurred in humans.

Flash point: 50°C

H314 - Causes severe skin burns and eye damage
H317 - May cause an allergic skin reaction
H370 - Causes damage to organs
H311 - Toxic in contact with skin
H331 - Toxic if inhaled
H301 - Toxic if swallowed
H351 - Suspected of causing cancer
H226 - Flammable liquid and vapor

Glacial acetic acid *(use under a fume hood)*

Eye: causes severe eye burns (with liquid or vapour)
Skin: May cause skin sensitisation. Causes severe burns with delayed tissue destruction.
Ingestion: May cause severe and permanent damage to the digestive tract.
Inhalation: May cause respiratory tract irritation with burning pain in the nose and throat, coughing, wheezing, shortness of breath and pulmonary oedema.

Flash point: 40°C

H314 - Causes severe skin burns and eye damage
H226 - Flammable liquid and vapor

Sodium hydroxide *(use under a fume hood when in solution)*

Eye: May cause eye irritation.
Skin: May cause skin irritation.
Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhea.
Inhalation: May cause respiratory tract irritation

H318 - Causes serious eye damage
H314 - Causes severe skin burns and eye damage