Guidance Document on Amphibian Thyroid Histology Part 1: Technical guidance for morphologic sampling and histological preparation

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Overall Objectives:

The goals of this document are to provide guidance on the collection of morphologic data and preparation of thyroid tissues, from *Xenopus laevis*, for histopathological analysis to fulfill the needs of the Amphibian Metamorphosis Assay. This document draws from the guidance developed for the "Histopathology guidelines for Phase 1B of the OECD Fish Screening Assay for EDC's" and the guidance given in the general protocol for the Phase 2 Amphibian Metamorphosis Assay. The recommended procedures were derived from pathologists experienced with preparing and analyzing thyroid tissues of *Xenopus laevis*, from previous documents relating to histotechniques associated with preparing amphibian tissues, and from the scientific literature. The procedures are to guide the handling of animals and preparation of tissues for thyroid histology. These methods may not apply to studies that involve other amphibian species, at earlier or later life stages, under alternate treatment durations, or for different desired endpoints.

This initial guidance document is divided into two sections: 1. Humane sacrifice and necropsy procedures and 2. Tissue preparation and histotechniques. Please see "Guidance Document on Amphibian Thyroid Histology Part 2: Approach to reading studies, diagnostic criteria, severity grading, and atlas" for information regarding approach to reading studies, evaluation of histopathologic diagnostic criteria, terminology and data recording.

I. Humane Sacrifice Procedures

The purpose of this section is to outline steps and procedures involved in humane sacrifice of *Xenopus laevis* tadpoles, necropsy technique, and fixation of tissues.

Objectives:

1. Humanely euthanize tadpoles
2. Obtain body weights, developmental stage and evaluate gross morphology
3. Acquire photodocumentation to determine hind limb lengths, whole body lengths, and snout-vent lengths
4. Provide for adequate fixation of the thyroids and the carcass

This section include:

A. Humane euthanasia of *Xenopus laevis* tadpoles
B. Tissue fixation and decapitation procedures

Recommended Materials:

1. Tadpole transport container (~500 ml, containing dilution water from the system reservoir).
2. Small mesh dip net.
3. Euthanasia chamber (~500 ml vessel).
4. Euthanasia solution (200 mg/L tricaine methanesulfonate (MS-222) appropriately buffered with sodium bicarbonate) (See Appendix 1).
5. Razor blade
6. Electronic analytical balance (minimum display: ≤ 0.1mg) and tarred vessels.
7. Davidson's fixative (See Appendix 1)
8. 10% neutral buffered formalin (See Appendix 1)
9. Appropriately sized pre-labeled plastic tissue cassettes (one per tadpole).
10. Fixation containers (15-20 mL, one per tadpole).

A. Humane euthanasia using MS-222 of *Xenopus laevis* tadpoles

Procedures:

1. Tadpoles should be sacrificed within one to two minutes prior to fixation. Therefore, unless multiple prosectors are available, numerous tadpoles should not be sacrificed simultaneously.
2. Using the mesh dip net, a tadpole is removed from the experimental chamber and transported to the necropsy area in the transport container.
3. The tadpole is placed into the euthanasia solution. The tadpole is removed from the solution when it is unresponsive to external stimuli.
4. Place the tadpole in a Petri dish containing euthanasia solution.
5. Determine the developmental stage according to Nieuwkoop and Faber (1994). *A single person should conduct all developmental stage determinations in a study to avoid inter-individual differences.
6. At this time, digital documentation is to be performed and notes made on any gross morphologic abnormalities. (Please see Appendix 2 for photo documentation details)

7. After digital documentation, blot the tadpole dry, weigh to the nearest milligram and measure whole body length and snout-vent length according to the protocol.
8. At this time, tadpoles that will be used for histological analysis are to be fixed.

B. Tissue fixation and decapitation procedures

It is recommended that tadpoles be fixed prior to decapitation. The advantage of decapitating the tadpole post-fixation is that the tissue becomes more rigid, and the decapitation can be done cleanly to facilitate sectioning. In addition, it will save time during the final sacrifice because decapitation can be performed at a later date. Finally, preliminary evidence suggests that complete fixation is obtained when the tadpole is submerged into Davidson's solution intact.

Procedures:

1. Euthanized whole tadpoles that are to be preserved for histology (approximately 50/chemical) are placed into individually labelled containers (50 mL tubes) with Davidson's fixative. The volume of fixative in the container should be at least 10 times the approximated volume of the tissues.
2. All tissues remain in Davidson's fixative for at least 48 hours, but no longer than 96 hours at which time they are rinsed in tap water and stored in 10% NBF.
3. Once fixed, the tadpole is placed in ventral recumbency and decapitated in a plane perpendicular to the caudal-rostral axis. To ensure that the decapitated tissue contains the thyroid gland, transect the carcass just rostral to the heart using a razor blade.

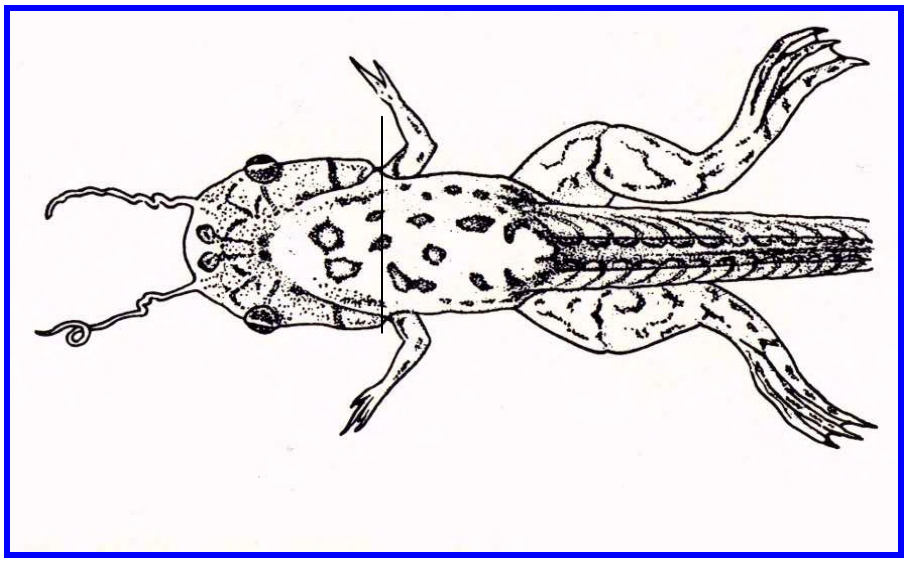


Figure 1. Site of decapitation.

4. Decapitated head tissue samples containing the lower jaw are placed in pre-labeled plastic cassettes and transferred to individual containers of 10% neutral buffered formalin [4% formaldehyde (NBF)]. Gently agitate the NBF for 5 seconds to dislodge any air bubbles that might adhere to the cassettes.

5. Place the remaining carcass into the container of NBF with the corresponding head tissue to facilitate tracking histological observations with developmental stage and hind limb data.

II. Histological preparation of tissues

The purpose of this section is to outline steps and procedures involved in histological preparation of tissues obtained from *Xenopus laevis* tadpoles.

Objectives:

1. Describe methods for trimming tissue
2. Describe tissue dehydration and embedding
3. Describe methods for microtomy

This section includes:

A. Tissue Trimming
B. Tissue Dehydration and Embedding
C. Microtomy
D. Staining, Coverslipping and Slide Labeling

A. Tissue Trimming

Either entire head sections, or mandibular sections with adjacent tissue, are acceptable tissues for embedding and microtomy. The tissues can be oriented ventral to dorsal on a horizontal plane in the mold to allow for sectioning of the ventral face first (frontal plane), or with the decapitation site oriented down, to accomplish transverse sectioning.

B. Tissue Dehydration and Embedding

Objectives:

1. Dehydrate tissue to provide for adequate penetration of paraffin.
2. Impregnate the tissue with paraffin to maintain tissue integrity and create a firm surface for microtomy.

Recommended Materials:

1. Tissue processor
2. Processing unit oven
3. Activated charcoal
4. Paraffin (Paraplast®, or equivalent)
5. 10% neutral buffered formalin. (See Appendix)
6. Ethyl alcohol (80%, 90%, 96%)
7. 100% isopropanol
8. Methyl benzoate
9. Embedding station (thermal, dispensing and cryo consoles)
10. Paraffin heating pots
11. Paraffin transfer pots

12. Thermometer
13. Embedding molds
14. Block drawers
15. Forceps
16. Scraper

Procedures:

1. A general dehydration, clearing and embedding procedure is listed below. Changes to this procedure that reflect differences in individual laboratory protocols are acceptable as long as laboratory protocols consistently provide high quality tissue sections. The tissue samples will be embedded in a manner that allows either the ventral surface of the tissues to be microtomed first (frontal sectioning), or the caudal portion of the head/mandibular tissue first (transverse sectioning). A routine *processor* protocol for dehydration and initial embedding is provided below. This protocol can be adapted for manual dehydration, clearing and embedding.

A.	80% EtOH	60 min	room temperature
B.	80% EtOH	60 min	room temperature
C.	90% EtOH	60 min	room temperature
D.	90% EtOH	60 min	room temperature
E.	96% EtOH	60 min	room temperature
F.	96% EtOH	60 min	room temperature
G.	100% isopropanol	60 min	room temperature
H.	100% isopropanol	60 min	room temperature
I.	methyl benzoate	60 min	room temperature
J.	methyl benzoate	12 hours	room temperature
K.	methyl benzoate	3 hours	room temperature
L.	paraffin I	3 hours	57°C (or 30 minutes at 65°C)
M.	paraffin II	12 hours	57°C (or 30 minutes at 65°C)

2. Labeled tissue cassettes are removed from formalin storage and are rinsed in tap water.
3. The cassettes are placed in the processing basket(s) in a single layer. The processing basket is loaded into the tissue processor and the dehydration, clearing and initial embedding processes are executed (A – M).
4. The cryo console of the embedding station is turned on. (Power to the dispensing console and thermal console should remain on at all times.)
5. The basket(s) of cassettes is/are removed from the processor and immersed in the paraffin-filled front chamber of the embedding station thermal console.
6. The first cassette to be embedded is removed from the front chamber of the thermal console. The cassette lid is removed and discarded, and the cassette label is checked against the animal records to resolve potential discrepancies prior to embedding.
7. An appropriately sized embedding mold is selected.
8. The mold is held under the spout of the dispensing console and filled with molten paraffin.
9. The tissues are removed from the base of the cassette and are placed in the molten paraffin in the mold. The tissues are oriented ventral to dorsal on a horizontal plane

in the mold to allow for sectioning of the ventral face first (place the ventral plane down such that it is on the leading edge of the block), or rostral to caudal to allow for transverse sectioning (placing the caudal portion of the head/mandibular tissue down so that it is on the leading edge of the block).

10. The base of the cassette is placed on top of the mold. Additional paraffin is added to cover the bottom of the base.
11. The mold with the cassette base is placed on the cooling plate of the cryo console.
12. After the paraffin has solidified, the block (i.e., the hardened paraffin containing the tissues and the cassette base) is removed from the mold.
13. Steps 3 through l0 are repeated for each cassette to be embedded.

C. Microtomy

Objective:

Create and mount histologic sections for staining.

Materials:

Microtome.
Disposable microtome knives.
Lipshaw Pike® oil (or equivalent lightweight, machine oil).
Temperature-controlled water bath.
Ice.
Microscope slides.
Staining racks.
Permanent slide marking pen.
Forceps.
Fine-tipped paint brush.
Temporary labels.
Slide warmer/oven.

Procedures:

1. The temperature in the water bath is allowed to stabilize so that ribbons cut from the tissue blocks will spread out uniformly on the surface without melting. This temperature assessment is a qualitative judgment made by the microtomist before and during microtomy. A good starting point is approximately 48-49°C.
2. If necessary, a new blade is mounted onto the microtome and the microtome is lubricated with oil.
3. The initial phase of microtomy is termed “facing” the block and is conducted as follows:
    a. The block is placed in the chuck of the microtome.
    b. The chuck is advanced by rotating the microtome wheel and thick sections are cut from the paraffin surface of the block until the knife reaches the embedded tissues. This process is referred to as “rough trimming” of the block.
    c. The section thickness on the microtome is set between 4 -10 microns. The chuck is advanced and multiple sections are cut from the block to remove any

artifacts created on the cut surface of the tissue during rough trimming. This process is termed “fast trimming” of the block.

d. The block is removed from the chuck and placed facedown on ice to soak the tissue (at the discretion of the microtomist).
e. Steps a through d are repeated until all blocks to be microtomed have been faced.
f. If it is determined during facing that any block is not of acceptable quality for microtomy (e.g. evidence of incomplete tissue infiltration), it is returned for re-embedding before proceeding with microtomy.
g. Any extraneous pieces of paraffin are removed from the microtome and workstation periodically during facing and before proceeding with the next phase of microtomy.

4. The next phase of microtomy is final sectioning and mounting of tissue sections on slides. These procedures are conducted as follows:
    h. Macroscopic lesions (if any) that are reported in the records are noted. Care is taken to include any macroscopic lesions in the sections collected during final sectioning.
    i. The block is removed from the ice and placed in the chuck of the microtome.
    j. With the section thickness on the microtome set to 5-7 microns, the chuck is advanced by rotating the microtome wheel. Initially, five step sections (30 microns apart) are taken from each block, and two serial sections of each step are placed on each of five slides. These slides are examined to assure that a sufficient amount of thyroid tissue is present bilaterally in at least two of the step sections. If necessary to meet this criterion, additional step sections can be cut from the block. Sections should ideally be acquired from central portions of the thyroid glands rather than peripheral areas to provide an accurate reflection of thyroid size. Serial sectioning rather than step sectioning may be necessary to acquire central sections of the thyroid glands. Sections are cut from the block until a “ribbon” containing at least two acceptable sections has been produced. As necessary during sectioning, the block may be removed from the chuck, placed on ice to soak the tissue, and replaced in the chuck.
    k. Each ribbon is floated flat on the surface of the water in the water bath. An attempt is made to obtain at least two sections with sufficient amount of thyroid tissue present bilaterally, from 30 microns apart in the ribbon, and which have no wrinkles and have no air bubbles trapped beneath them.
    l. A microscope slide is immersed beneath the best sections in the floating ribbon. The sections are lifted out of the water using the slide. This process is referred to as “mounting” the section on the slide.
5. With a slide-marking pen, the block number from which the slide was produced is recorded on the slide along with a sequential number indicating the order in which the sections were obtained.
6. The slide is placed in a staining rack.
7. The block is removed from the chuck and placed facedown for storage.
8. Steps a through h are repeated for all blocks to be microtomed.

Notes:

To ensure that the thyroid glands have been obtained while sectioning, it may be necessary to examine the sections under a microscope prior to staining. The landmarks that are generally used for transverse sections include the eyes bilaterally and hyoid cartilage in the dorsal third of the section for transverse sectioning, however landmarks for frontal sectioning have yet to be described.

D. Staining, Coverslipping, and Slide Labeling

Objectives:

1. Differential staining of intra- and inter-cellular components of the thyroid glandss to facilitate diagnostic examination by brightfield microscopy.
2. Permanently seal mounted and stained tissues.
3. Permanently identify stained sections in a manner that allows complete traceability.

Materials:

1. Automated slide stainer (optional)
2. Robot coverslipping machine (optional)
3. Orange terpene
4. 0.1% Eosin-Y
5. Hematoxylin
6. Xylene
7. Absolute ethyl alcohol (100% ETOH)
8. ETOH (96%, 90%, 80%, 70%)
9. 100% isopropanol
10. H_2O
11. Coverslipping mountant (Permount or equivalent)
12. Glass coverslips, No. 1, 24 x 50 (or 60) mm
13. Slide flats

Procedures:

1. Staining
    A. Slides are routinely air-dried overnight before staining.
    B. Harris' H&E Staining protocol

a.	orange terpene	3 x 5 min
b.	100% isopropanol	2 x 5 min
c.	96% EtOH	3 x 3 min
d.	90% EtOH	3 x 3 min
e.	80% EtOH	3 x 3 min
f.	70% EtOH	3 x 3 min
g.	H_2O	2 min
h.	hematoxylin	15 min
i.	running H_2O	10 min
j.	0.1% eosin Y	3 min
k.	H_2O	short
l.	70% EtOH	short
m.	80% EtOH	short

n.	90% EtOH	short
o.	96% EtOH	1 min
p.	100% isopropanol	2 x 5 min
q.	orange terpene	3 x 5 min

C. A similar schedule can be adapted for manual staining. Differences in procedures that are reflected in individual laboratory protocols are acceptable as long as laboratory protocols consistently provide well stained sections with adequate contrast.

2. Coverslipping
   A. Coverslips can be applied manually or automatically.
   B. A slide is dipped in xylene, and the excess xylene is gently knocked off the slide.
   C. Approximately 0.1 ml of mounting medium is applied near the end of the slide opposite to the frosted end.
   D. A coverslip is tilted at a shallow angle as it is applied to the slide.
3. Labeling

   Each slide label should contain the following information:

Laboratory name
Species
Specimen No. / Slide No.
Chemical / Treatment group
Date (optional)

Appendix 1: Solutions

Euthanasia Solution:

200 mg/liter MS-222 appropriately buffered with sodium bicarbonate at 0.42 -1.05 g/liter. The goal is a pH of approximately 7.0. The characteristics of the water used to make this solution may impact the pH, therefore no exact recipe exists. However, begin using a recipe of one part MS-222 (150 mg/L of MS-222) plus two parts of sodium bicarbonate (300 mg/L of NaHCO3).

*Unbuffered MS-222 solution is irritating to frog skin and poorly absorbed resulting in a prolonged induction time.

Davidson's Fixative*:*

Formaldehyde (37-40%)	200 ml
Glycerol	100 ml
Glacial acetic acid	100 ml
Absolute alcohol	300 ml
Distilled water	300 ml

10% Neutral Buffered Formalin:

Formalin, full strength (37-40% formaldehyde)	100.0 ml
Sodium phosphate dibasic (anhydrous)	6.5 g
Sodium phosphate monobasic	4.0 g
Distilled water	900 ml

Appendix 2: Digital Documentation

Computer software analysis of digital images has been successfully applied in diverse biological research (Johnson et al., 2001; Machado-Silva et al., 2000; Merris et al., 2003), and is more accurate that the traditional caliper method. The following describes one procedure for acquiring digital photographs for measuring tadpole whole body length, snout-vent length, and hind limb length.

Procedure 1: Stereomicroscope

Note: Please refer to individual laboratory standard operating procedures for more detailed instructions.

Equipment:

1. Stereomicroscope equipped with a digital camera with micro function (e.g. Canon, EOS 10D, 4 mega pixels)
2. Lighting system (e.g. LEICA, CLS 150X)
3. Metric ruler
4. Image analysis software (e.g. Image Pro® Plus, Version 4.0, Media Cybernetics, U.S.A.) 1. Digital camera with micro function (e.g. Canon, EOS 10D, 4 mega pixels)

Procedure:

1. Place tadpole in a central position on the stage of the stereomicroscope in dorsal recumbency.
2. Place a calibration metric ruler and identifying number within the camera view next to the tadpole. Switch on lamps so that it illuminates the tadpole.
3. Two images of each tadpole should be acquired. One should be taken at a sufficiently low magnification to encompass the entire organism in order to document the developmental stage. The second should be taken at a higher magnification to be used in hindlimb measurements. When taking hindlimb photographs and measurements, it is suggested that one limb be analyzed consistently from specimen to specimen (e.g left hind limb only).
4. Adjust the focus prior to each photograph such that the hind limbs and metric scale are in full view.
5. Save the image files electronically. Each image file name should contain the identifying number.
6. Analyze digital images as described below.
7. If desired, lateral-view photographs of sampled tadpoles can also be taken to provide a means to electronically document length measurements. Calibration with a metric ruler as described above is needed for consistency.

Procedure 2: Glass board method

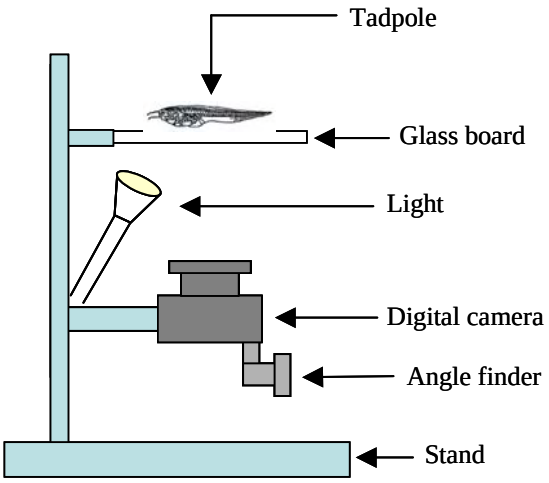
Equipment:

1. Digital camera with micro function (e.g. Canon, EOS 10D, 4 mega pixels)
2. Remote switch (e.g. Canon, RS-80N3)
3. Glass board (15 cm x 15 cm).
4. Lens (e.g. Canon, Macro Photo Lens MP-E 65 mm F2.8 1-5x)
5. Lighting system (e.g. LEICA, CLS 150X)
6. Image analysis software (e.g. Image Pro® Plus, Version 4.0, Media Cybernetics, U.S.A.)
7. Metric ruler

Procedure:

1. Attach the glass board to the stand.
2. Attach the camera to the stand such that the lens faces upward and under the glass board (see Figure 2).
3. Adjust the light under the glass board.
4. Attach the remote switch and the angle finder to the camera (Figure 1).
5. Wet the glass board slightly and place the tadpole carefully on the wet glass such that the ventral side faces the camera lens.
6. Place a calibration metric ruler and identifying number within the camera view next to the tadpole. This identifying number should be included in the image file name.
7. Switch on lamp so that it illuminates the tadpole from below.
8. Two images of each tadpole should be acquired. One should be taken at a sufficiently low magnification to encompass the entire organism in order to document the developmental stage. The second should be taken at a higher magnification to be used in hindlimb measurements.
9. Adjust the focus prior to each photograph such that the hind limbs and metric scale are in full view.
   *If measuring bent hind limbs is difficult, one can hyper-extend the hind limb as long as a deep anesthetic plane has been achieved.
10. Save each image electronically. Include the identifying number in the file name.

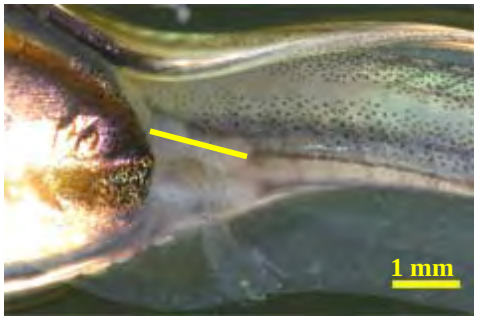
1. **Camera setup**



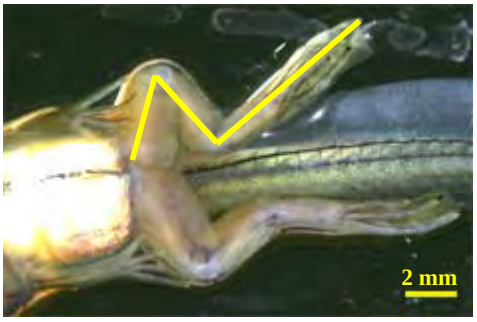
2. **System schematic**

Image analysis

1. Download the images from the camera to a computer. Use appropriate image-analyzing software for analysis of the digital images (e.g. Image-Pro® Plus).
2. Select the best images for measurements of the body length and the hind limb length, respectively.
3. For body length measurements, locate the tip of the snout, the end of the vent and the tip of the tail. Draw two lines, one from the tip of the snout to the end of the vent (snout-vent length) and another from the tip of the snout to the tip of the tail (whole body length).
4. For hind limb length measurements, locate the shining gold-colored part of the abdomen where the limb attaches to the abdomen, and draw a line right to the tip of the limb (Figure 3A).
5. If the limb is bent, draw the line from that part of the abdomen to the knee joint (Figure 3B). Click and create a node at the knee joint, and continue drawing the line all the way to the tip of the limb (Figure 3B).
6. Calculate the limb length accordingly.



3. **Hindlimb measurment – straight limb**



4. **Hindlinb measurement - bent limb**

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