TiLV Diagnostics: Histopathology and Collection of Sample (Level 2)

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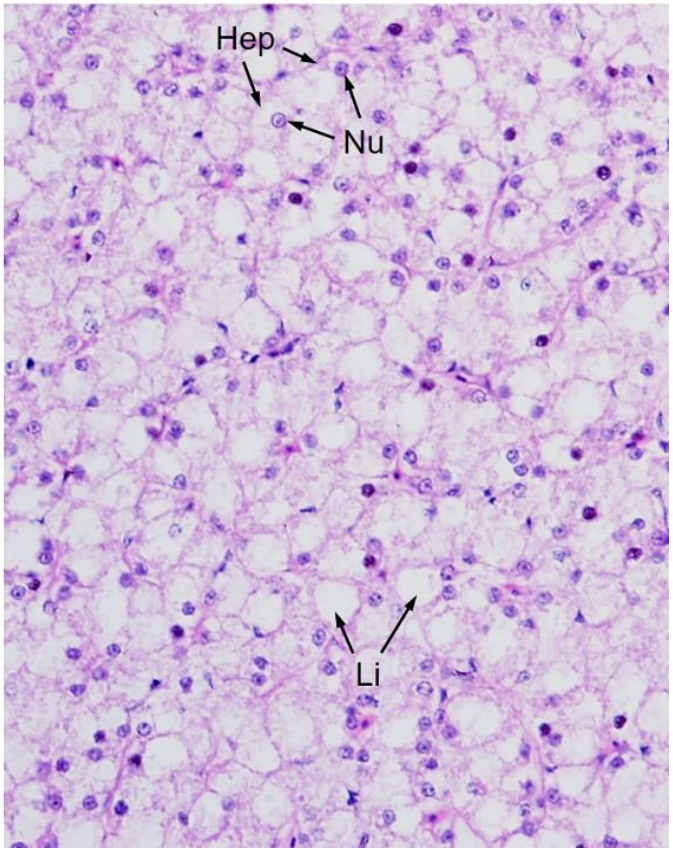
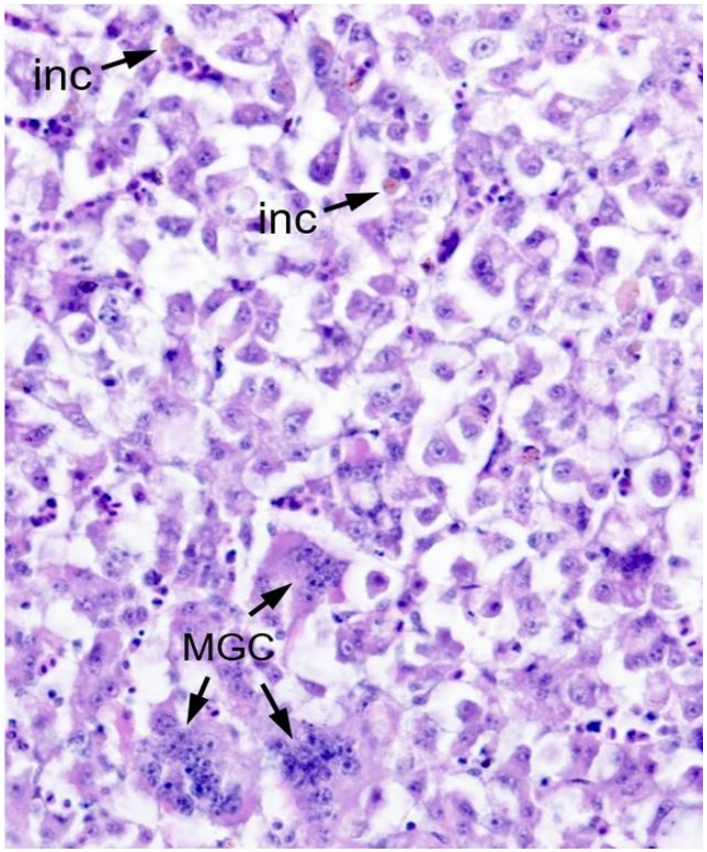
What is histopathology?

The study of changes in tissues caused by disease



Liver of normal fish



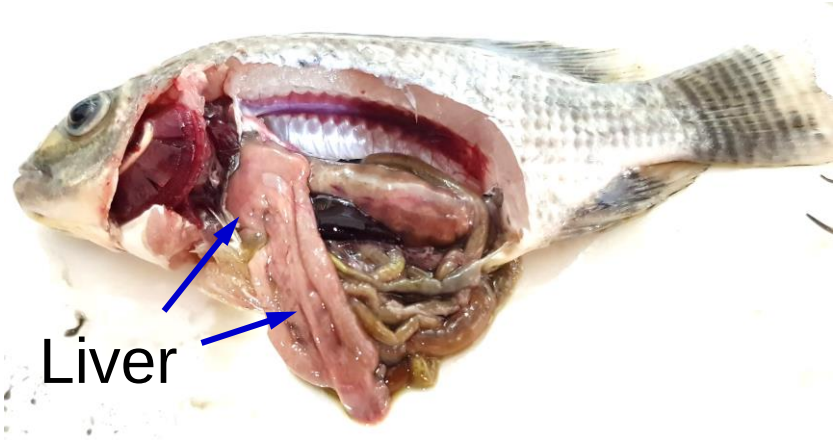
Liver of sick fish

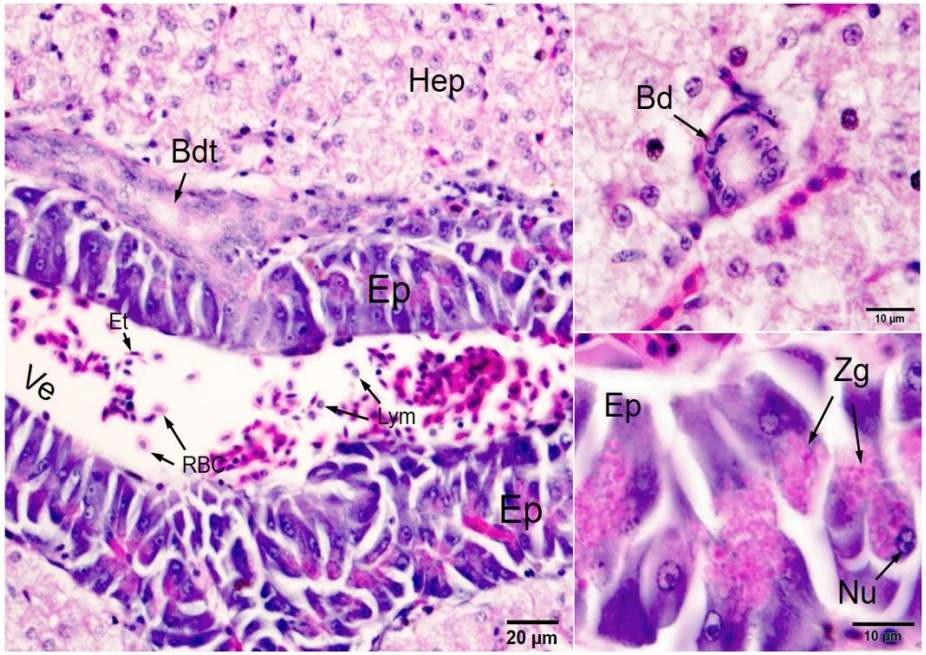
What is typical TiLV lesion?

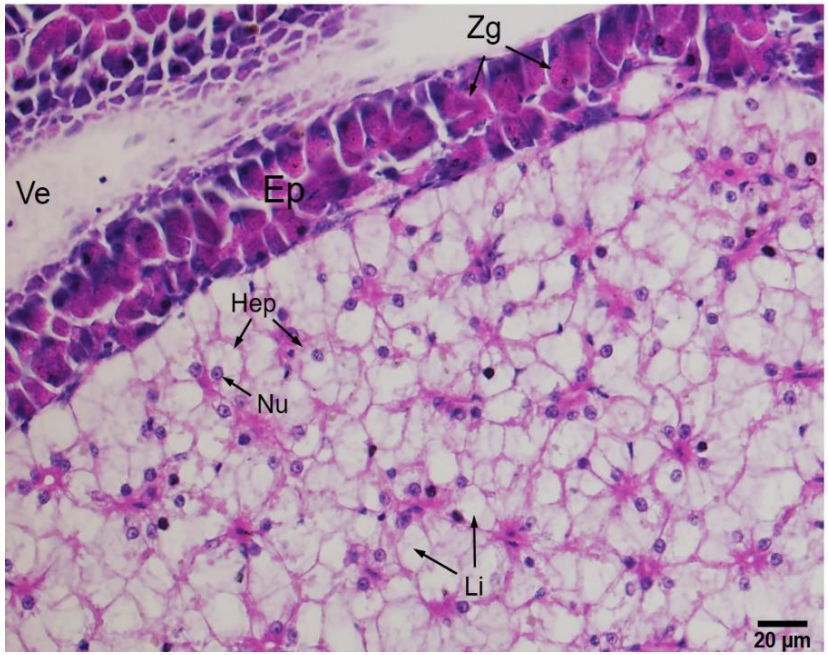
"Currently available information suggests syncytial hepatitis to be the most common histopathological feature found in TiLV outbreaks"

Mona et al. 2018 Review in Aquaculture

Normal Liver

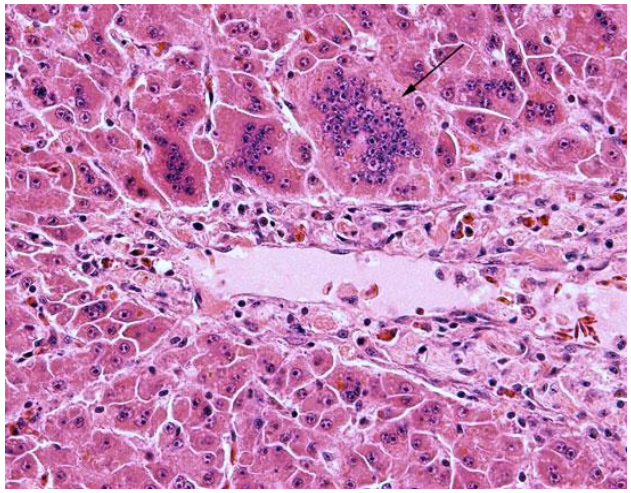




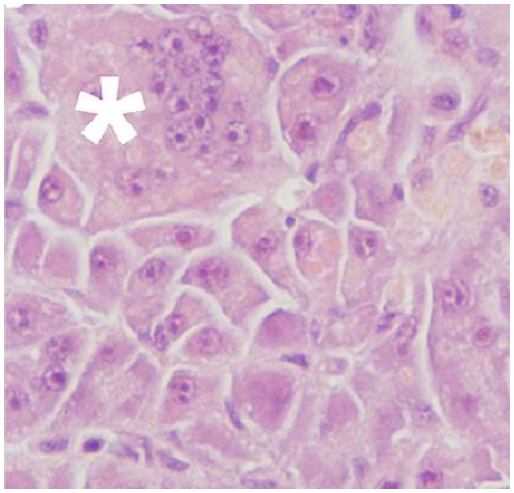


Photomicrographs of the H&E stained normal liver of Nile tilapia juvenile. Normal liver cells have polyhedral shape. Bdt, bile ductile; Ep, exocrine pancreas; Hep, hepatocyte; Li, lipid droplets; Nu, nucleus; RBC, red blood cells; Si, sinusoid; Ve, vein; Zg, zymogen granules

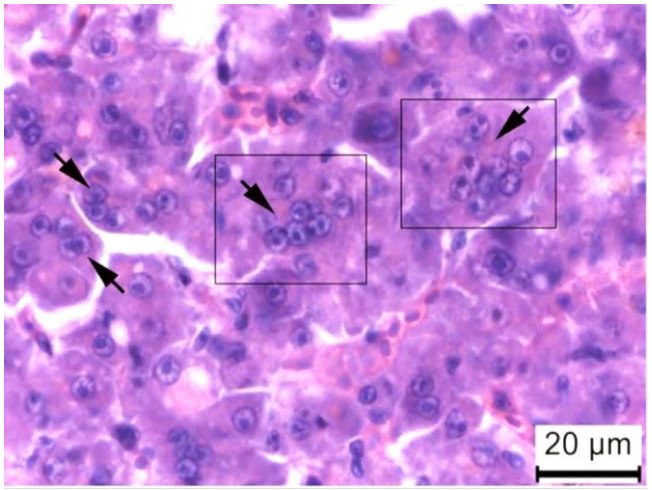
Typical histopathological lesion



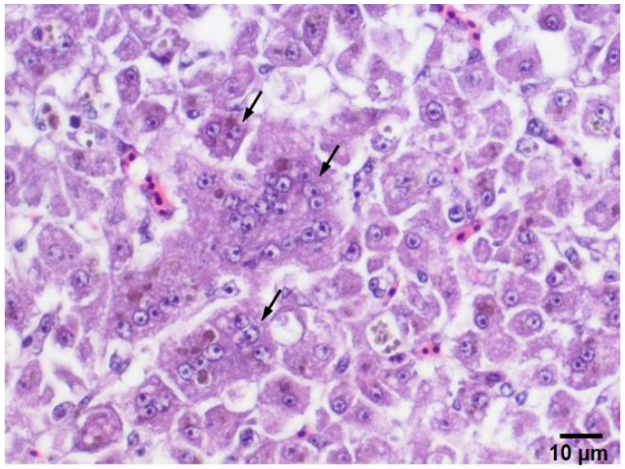
Ecuador (Ferguson et al. 2013)



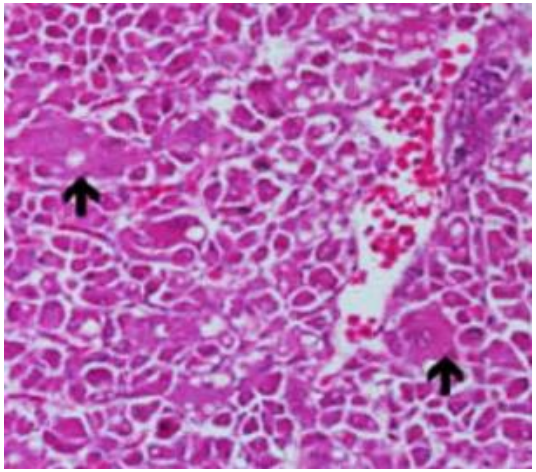
Israel (Bacharach et al. 2016)



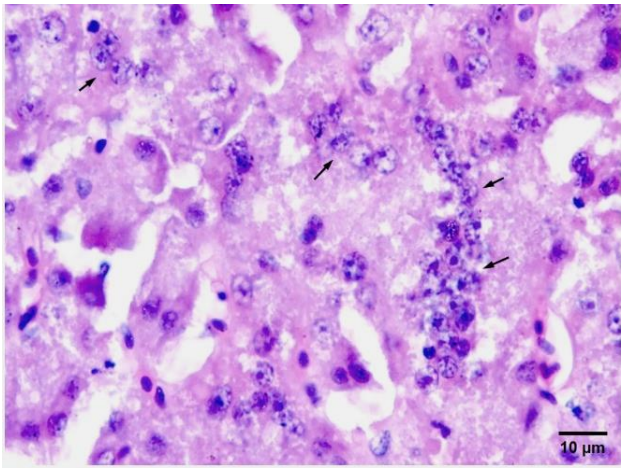
Thailand (Dong et al. 2017)



India (Behera et al. 2018)

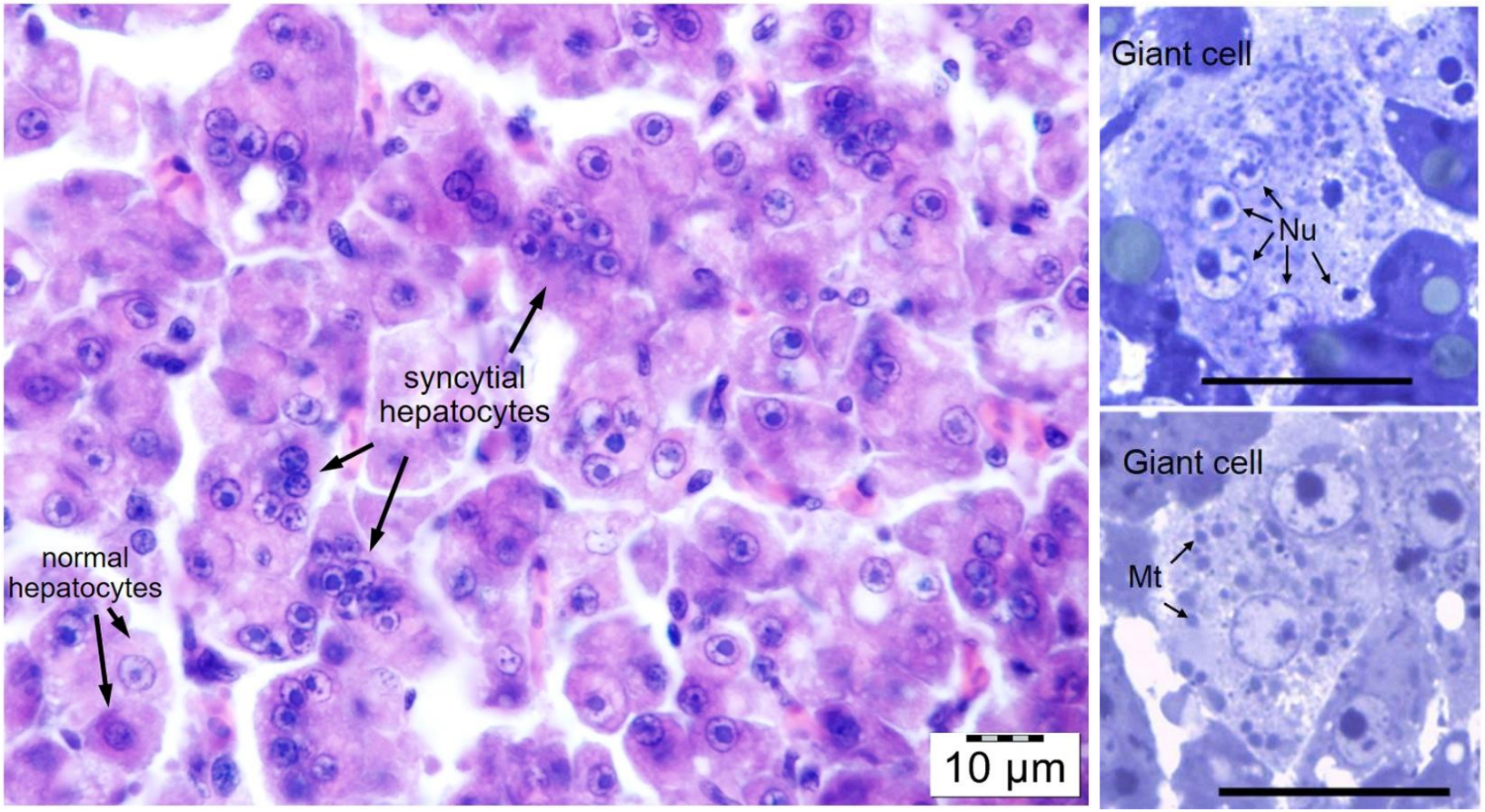


Malaysia (Amal et al. 2018)

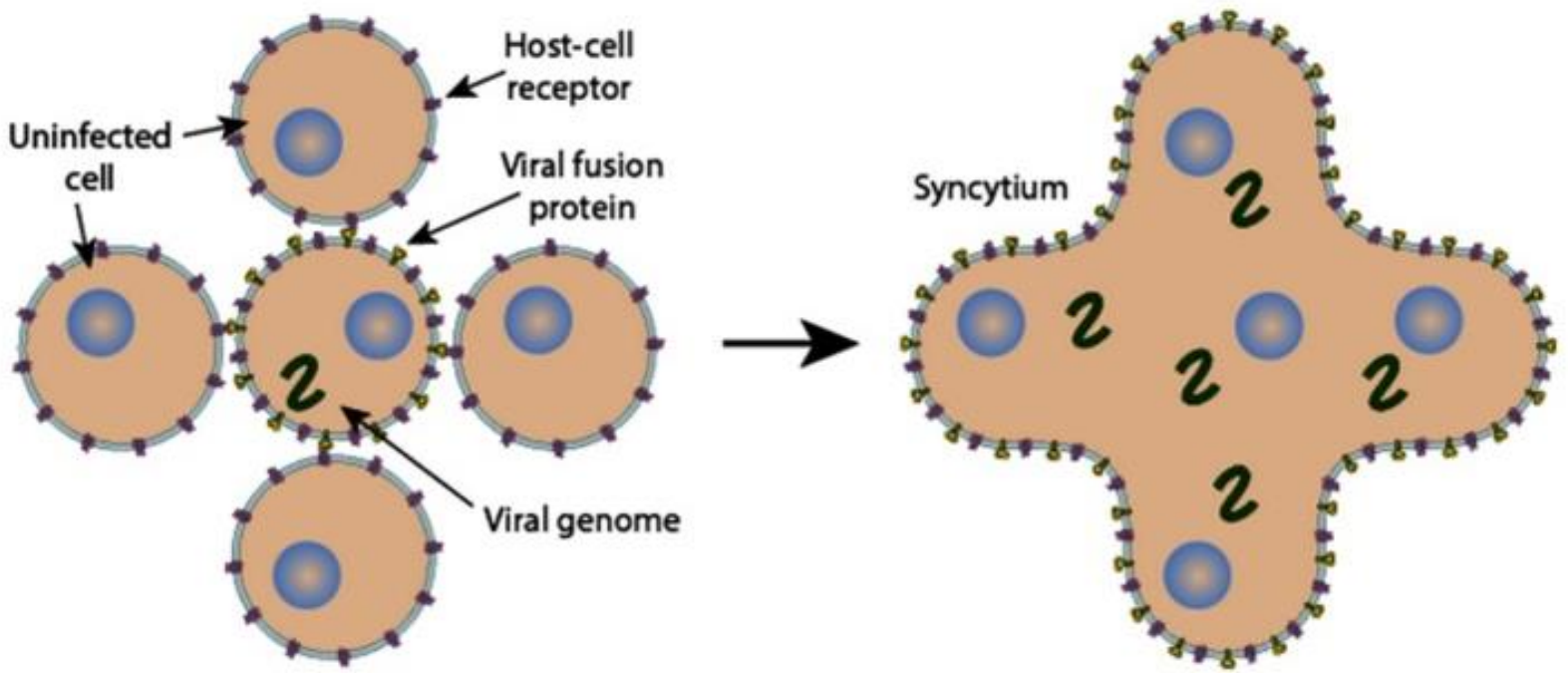


Peru (collected in 2018)

Typical histopathological lesion



Possible explanation for SHT



Source: www.expasy.org/viralzone

Other histopathological changes

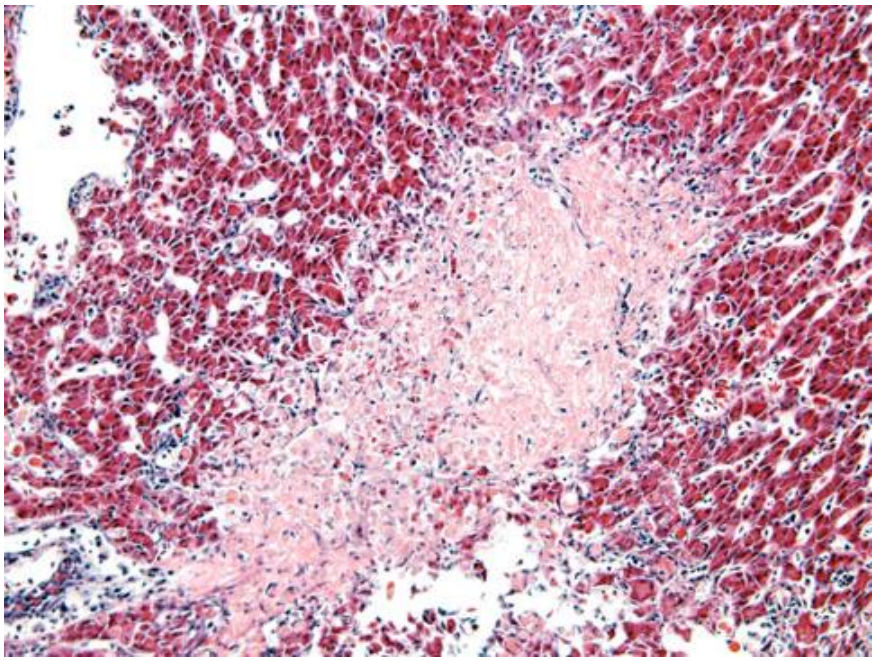
Liver

Atypical lesions (individual or combination of following lesions):

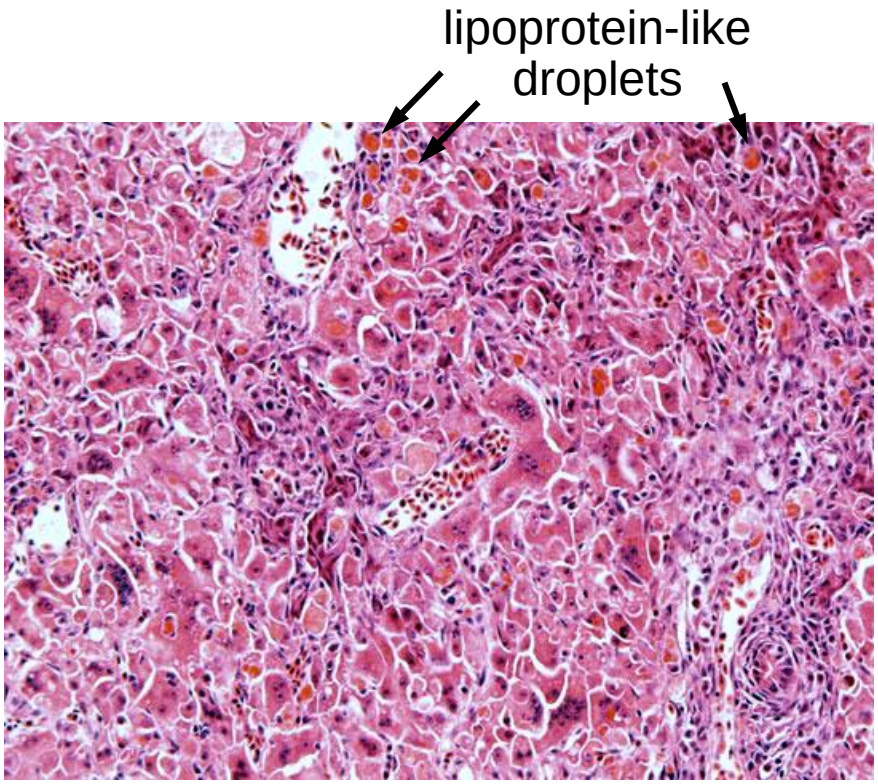
- ✓ multifocal chronic hepatitis
- ✓ presence of intracytoplasmic inclusion bodies (eosinophilic inclusion or lipoprotein droplets)
- ✓ reduction of fat-storage cells
- ✓ hepatocyte disassociation
- ✓ necrotic pancreases and infiltration of lymphocytes
- ✓ hemorrhage
- ✓ cellular necrosis
- ✓ pyknosis and karyorrhexis
- ✓ foamy cytoplasm

Other histopathological changes

Liver



multifocal chronic hepatitis

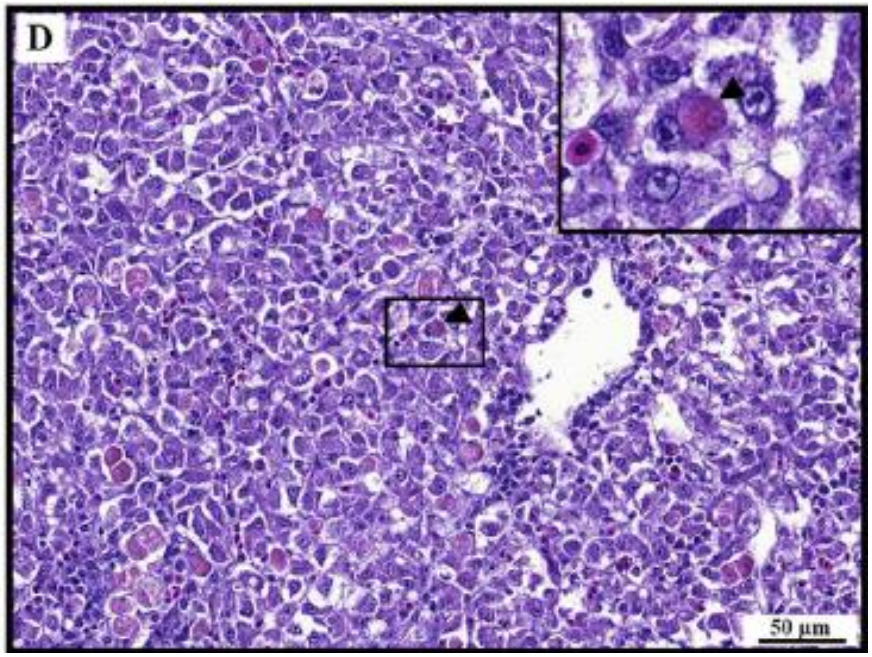


hepatocytes often containing
lipoprotein-like droplets

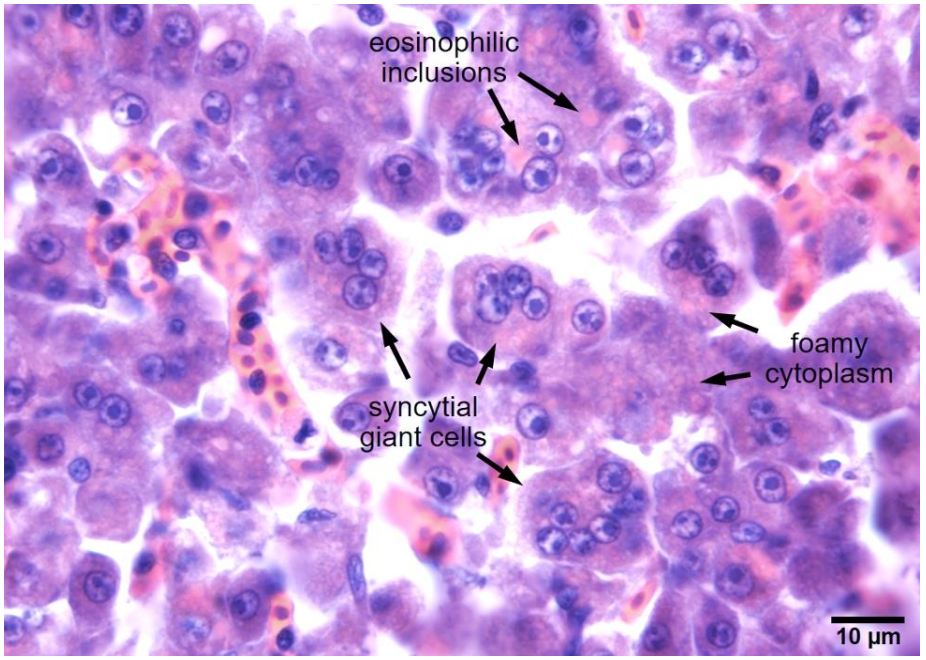
Ferguson et al. 2013

Other histopathological changes

Liver



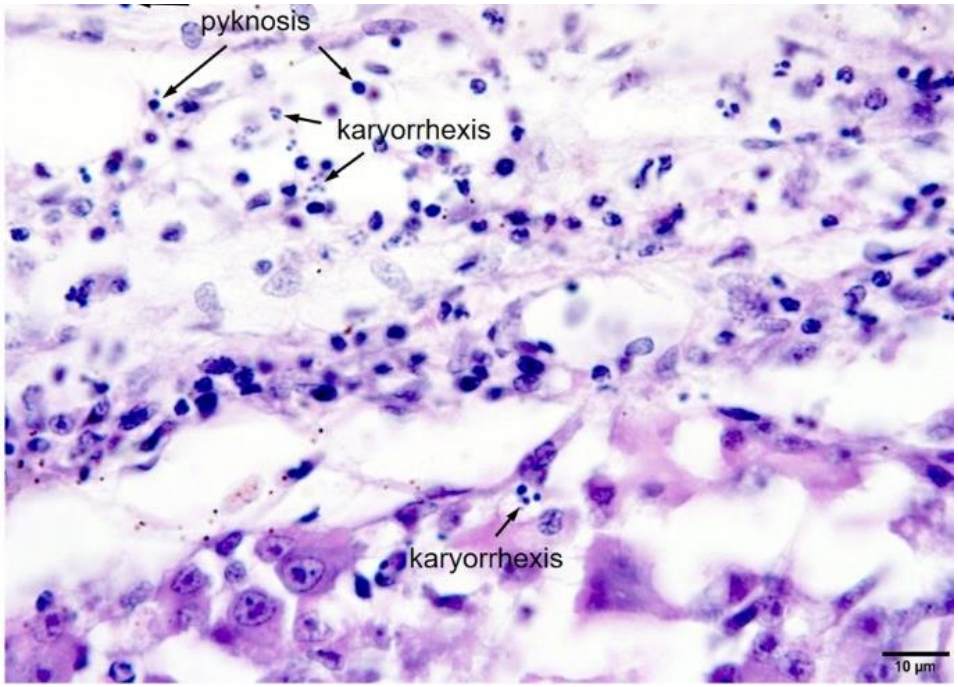
Intracytoplasmic inclusion bodies
Tattiyapong et al. 2017

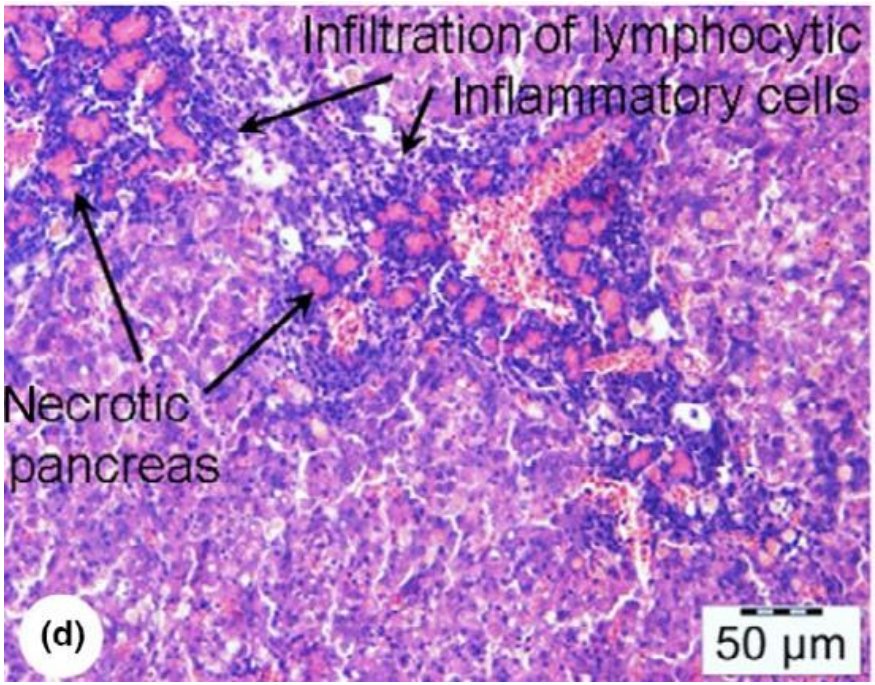


Syncytial giant cells, intracytoplasmic inclusion bodies, foamy cytoplasm
(HT Dong)

Other histopathological changes

Liver

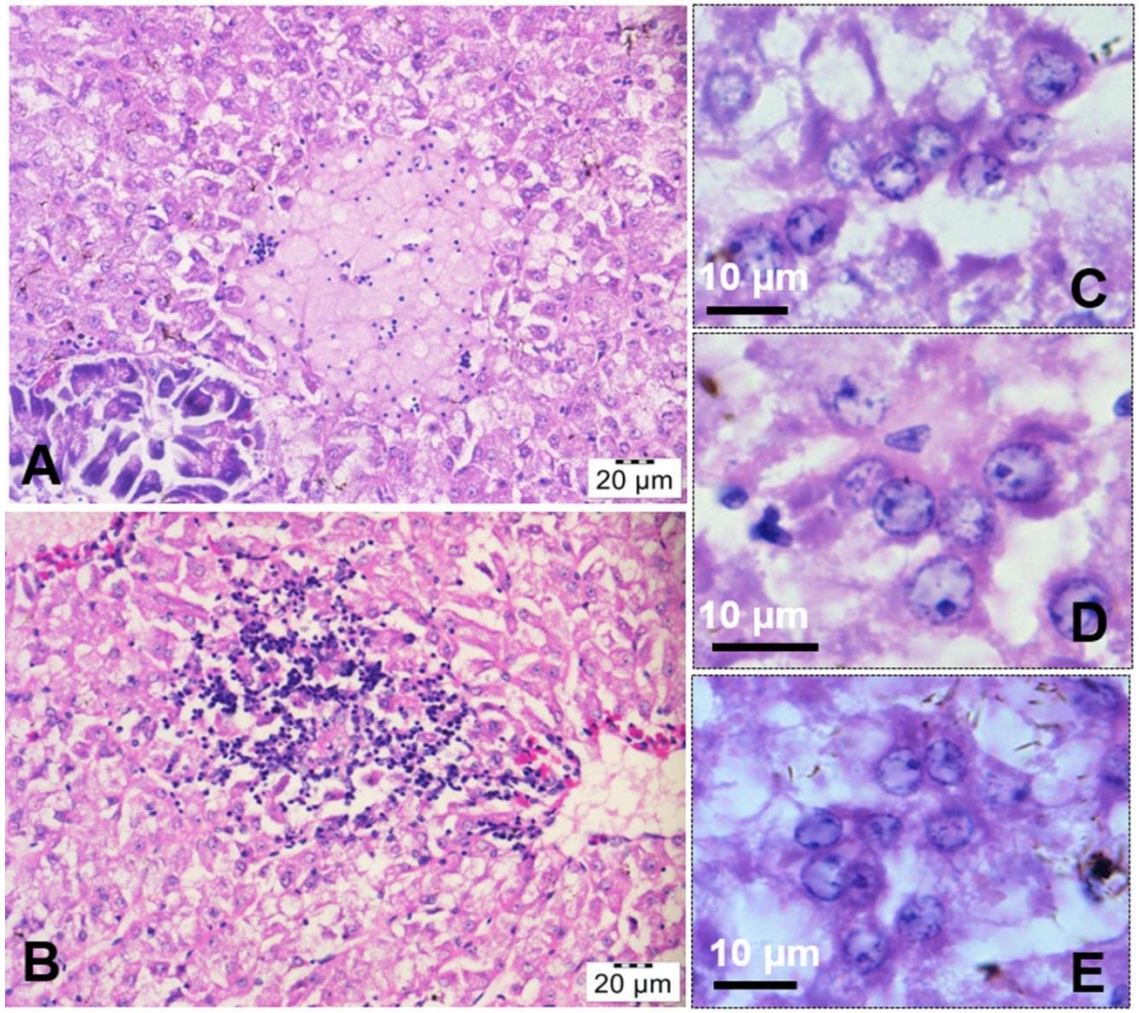




Subclinical infection

focal necrosis of hepatocytes

infiltration of lymphocytic inflammatory cells

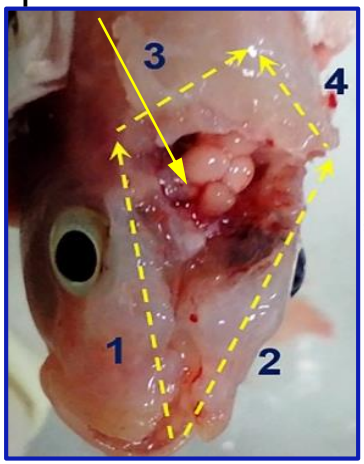


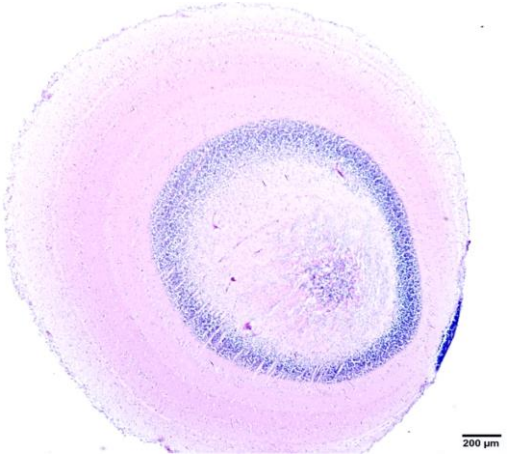
hepatocytes resembling giant cells which contained multiple nuclei

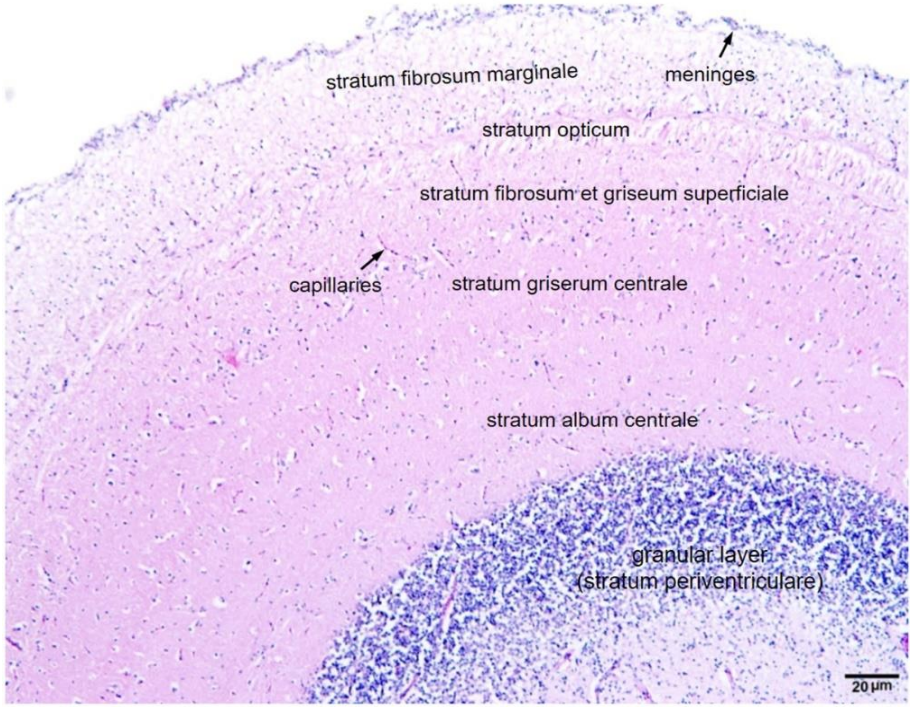
Senapin et al. 2018 Aquaculture

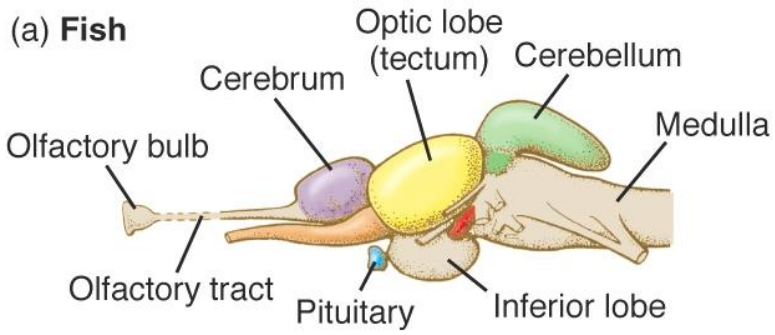
Normal Fish Brain

optic lobe





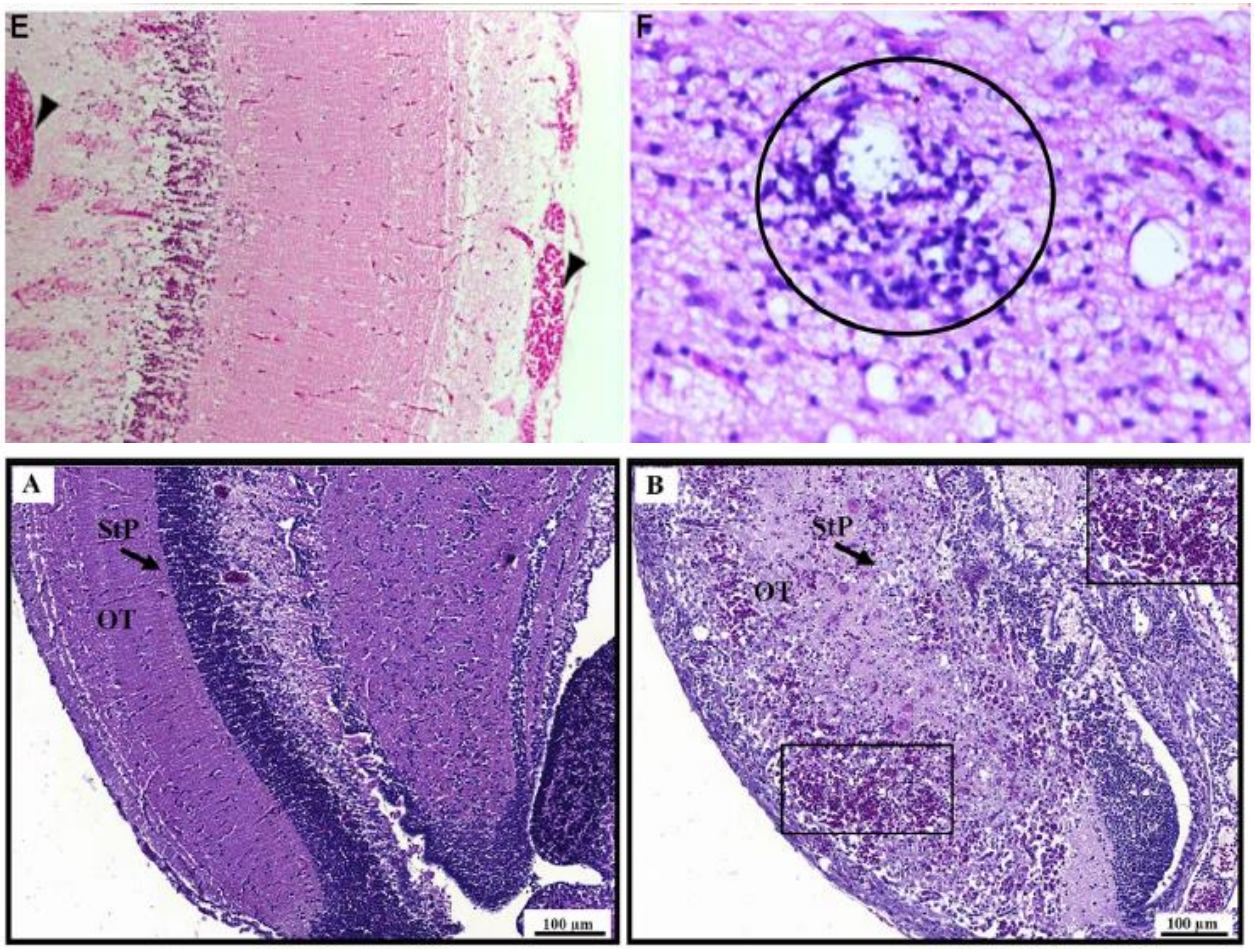




H&E stained optic tectum of the normal brain of tilapia

https://infograph.venngage.com/p/180563/science

Histopathological changes in the brain

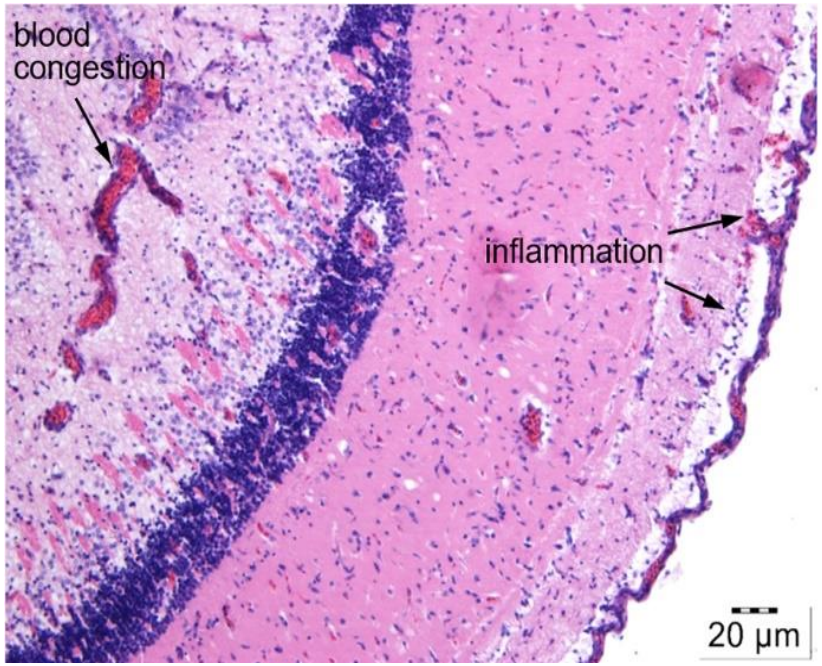


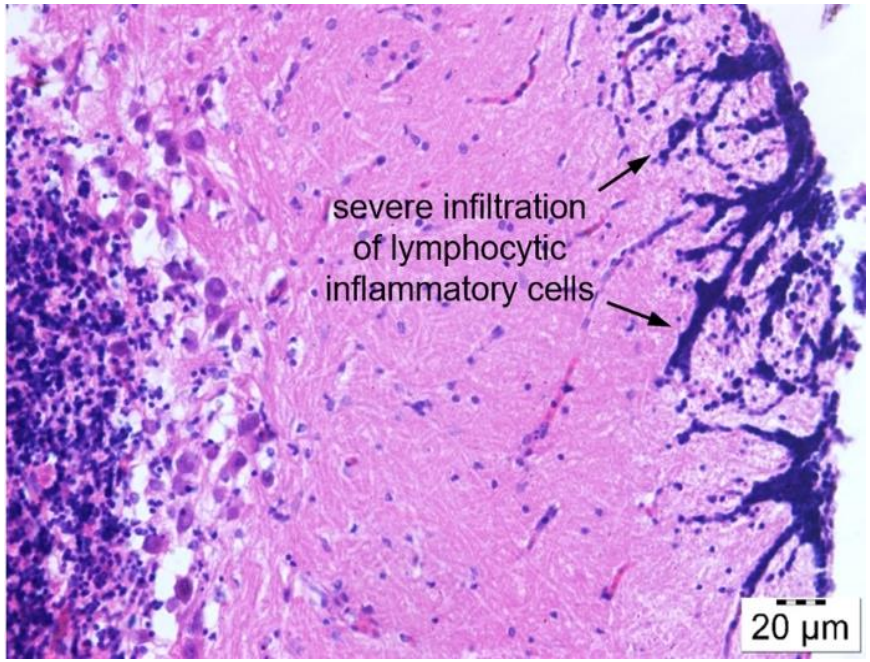
- ✓ congestion
- ✓ perivascular cuffing of lymphocytes in the brain cortex

- ✓ multifocal hemorrhage & blood congestion

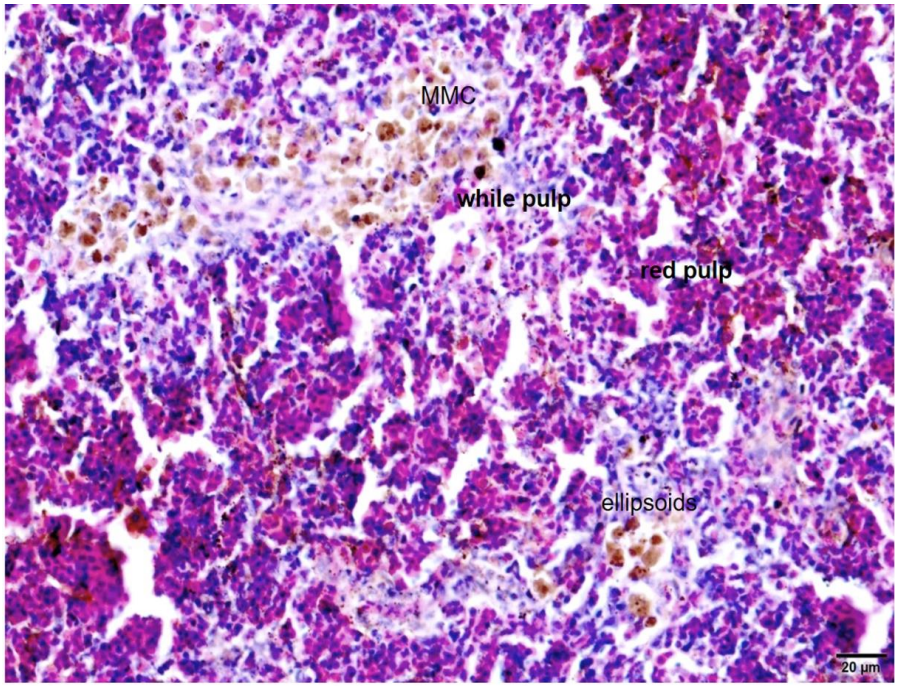
Eyngor et al. 2014; Tattiyapong et al. 2017

Histopathological changes in the brain

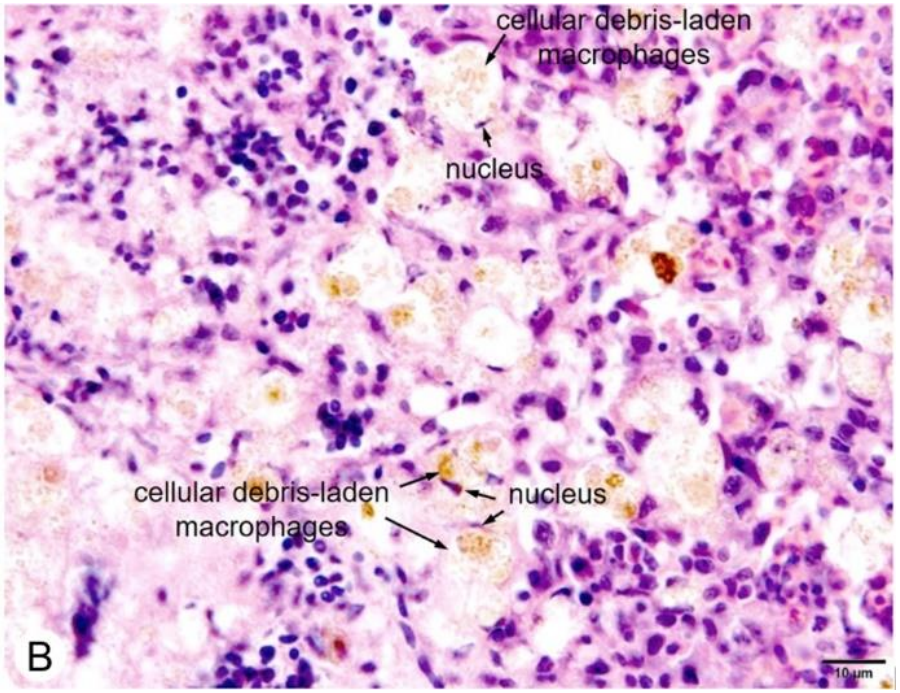




Histopathological changes in the spleen

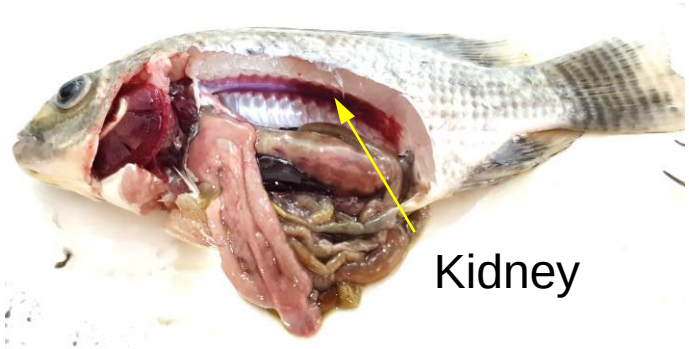


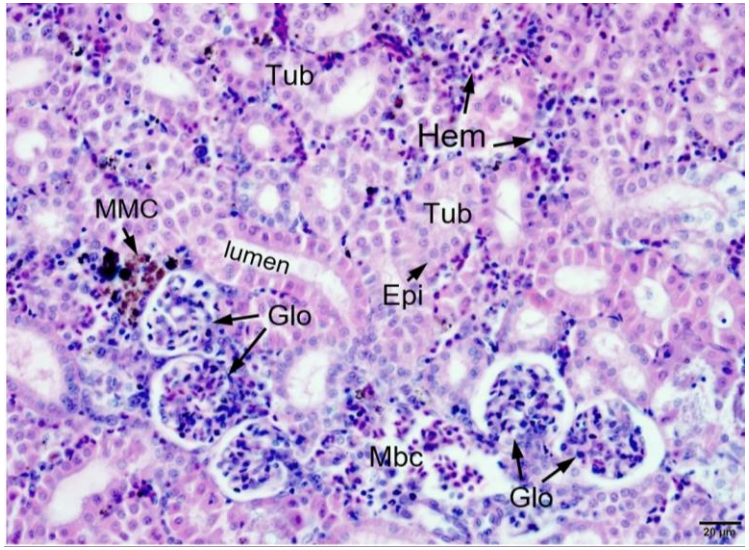
Normal spleen

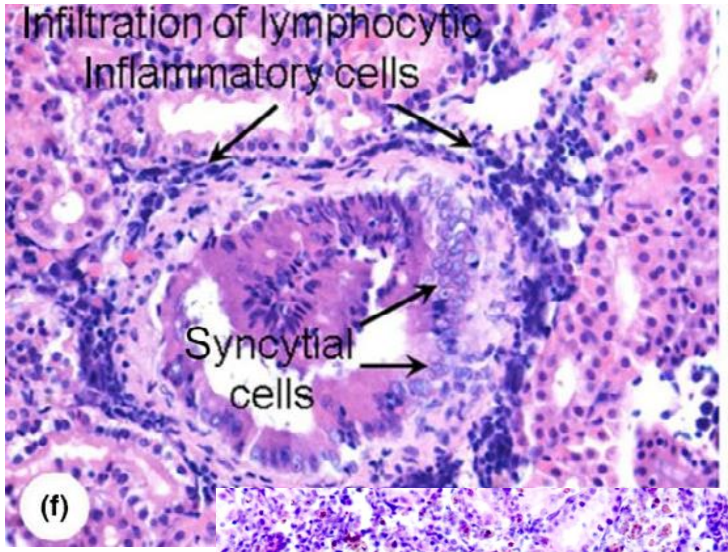


TiLV-infected spleen

Histopathological changes in the kidney

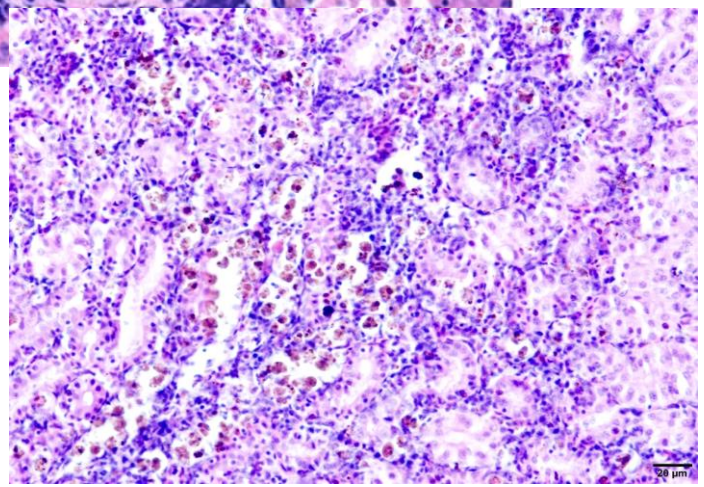




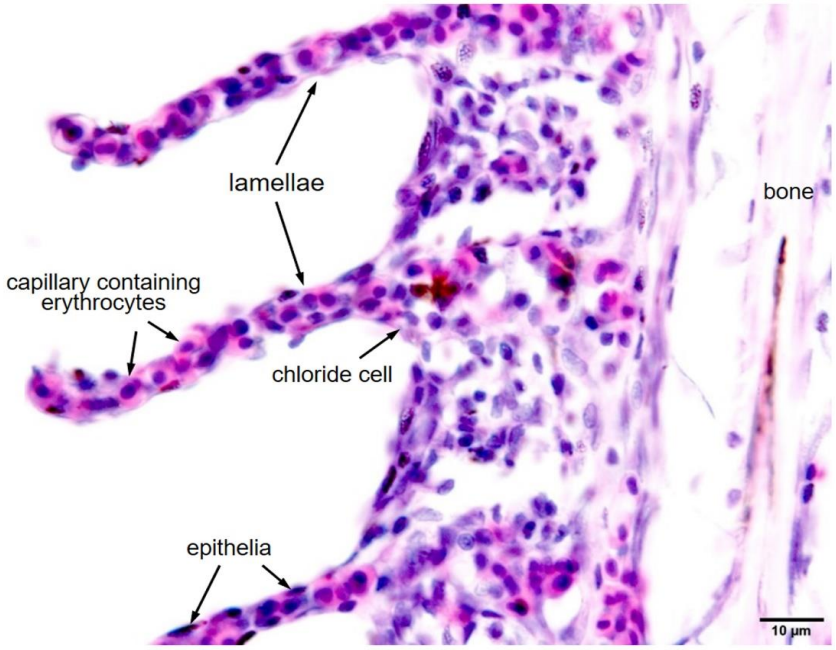


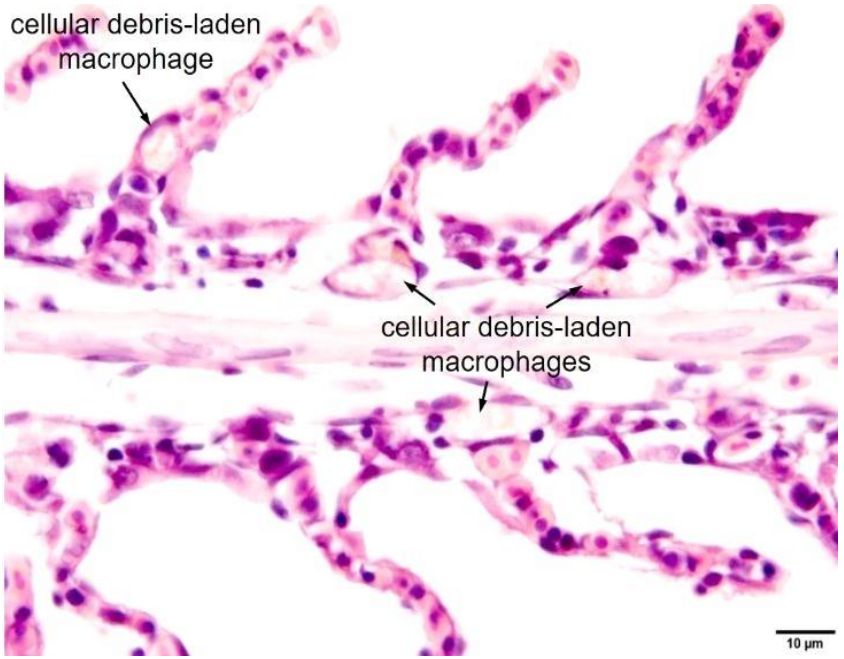
Normal kidney. Epi, epithelial cell; Glo, glomerulus; Hem, hematopoietic tissue; Mbc, mature blood cells; MMC, melano-macrophage center; Tub, tubules

increasing number of MMCs



Histopathological changes in the gills

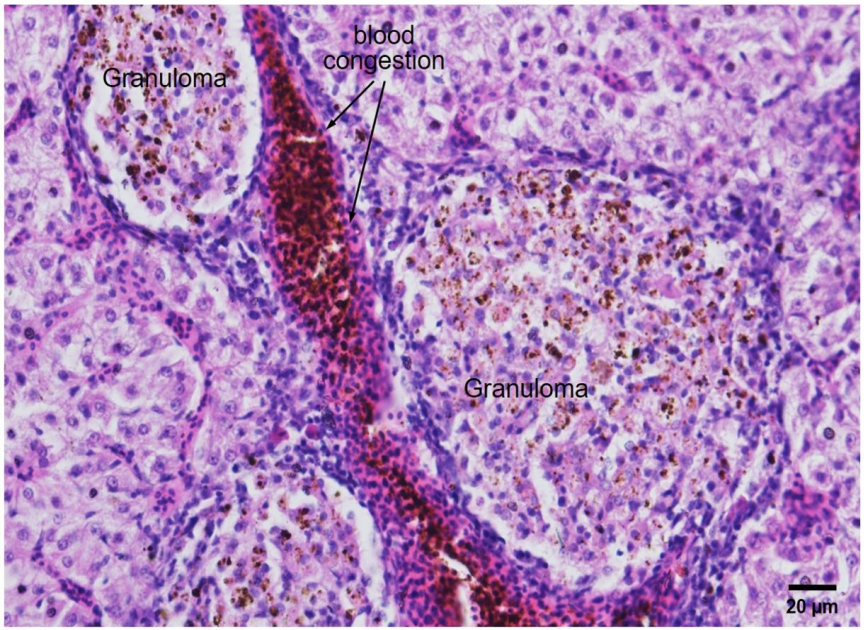




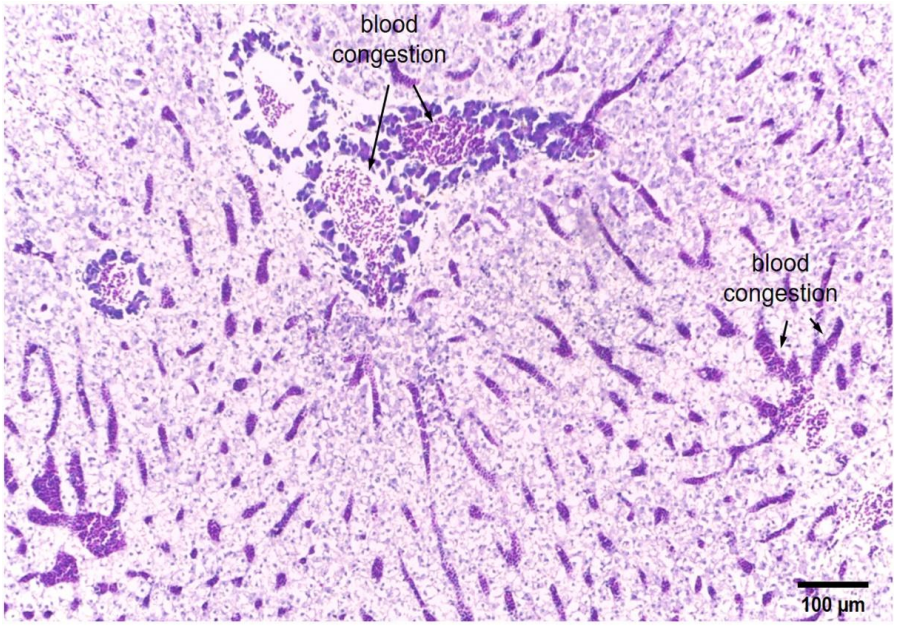
Organ	Histopathology description
Liver	**Typical lesion:** presence of syncytial giant cell(s) or multinucleated giant cells. **Atypical lesions** (individual or combination of following lesions): presence of intracytoplasmic inclusion bodies (eosinophilic inclusion or lipoprotein droplets), reduction of fat-storage cells, hepatocyte disassociation, necrotic pancreases and infiltration of lymphocytes, hemorrhage, cellular necrosis, pyknosis and karyorrhexis, foamy cytoplasm, multifocal chronic hepatitis.
Kidney	**Typical lesions:** none **Atypical lesions:** aggregation of lymphocytes, pyknosis and karyorrhexis, increasing number of melano-macrophages centers. Syncytia-like was occasionally seen.
Spleen	**Typical lesions:** none **Atypical lesions:** splenic cell degeneration, presence of debris-laden macrophages within splenic ellipsoids, pyknosis and karyorrhexis, increasing number of melano-macrophage centers.
Brain	**Typical lesions:** none **Atypical lesions:** severe inflammation with infiltration of massive lymphocytes, encephalitis, perivascular cuffing, blood congestion or sometime hemorrhage, syncytia-like was occasionally seen.
Gills	**Typical lesions:** none **Atypical lesions:** infiltration of lymphocytic inflammatory cells, pyknosis and karyorrhexis, presence of debris-laden macrophages.

Histopathology of other infections

Histopathology of other infections

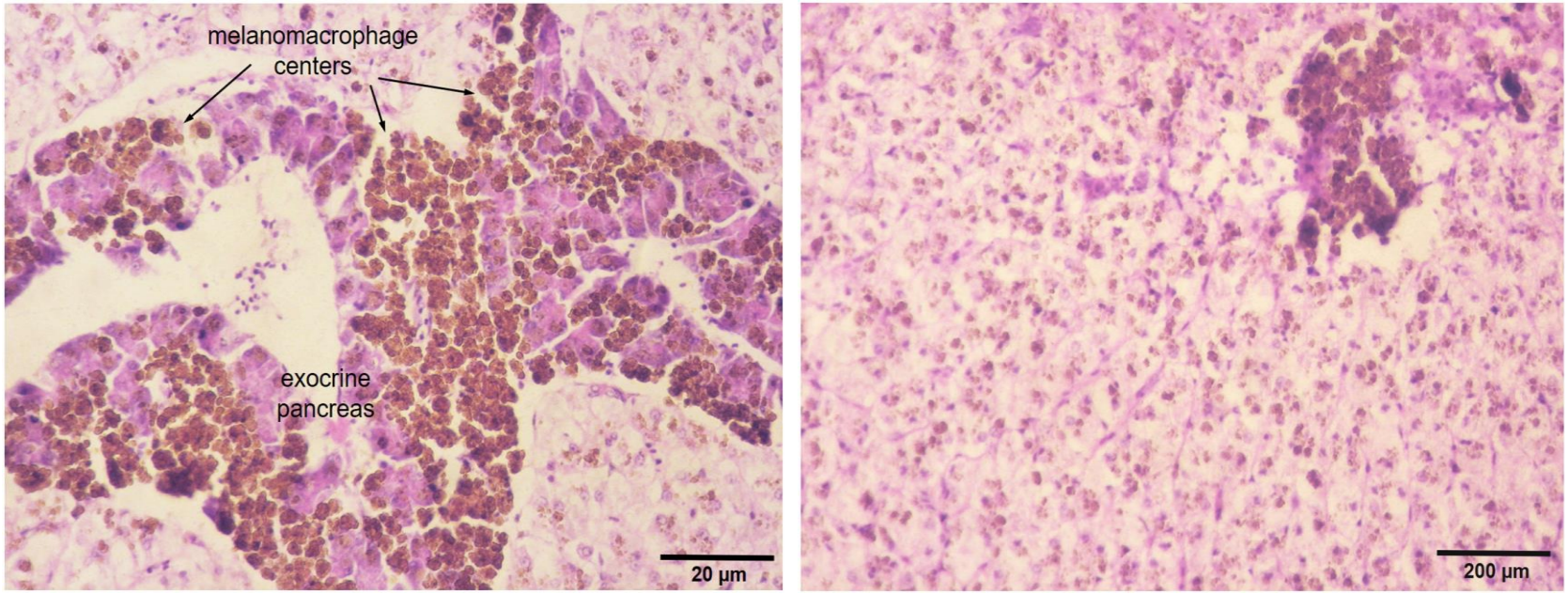


Liver of a hybrid red tilapia juvenile infected with *Francisella noatunensis* subsp. *orientalis* revealed typical granulomatous necrosis



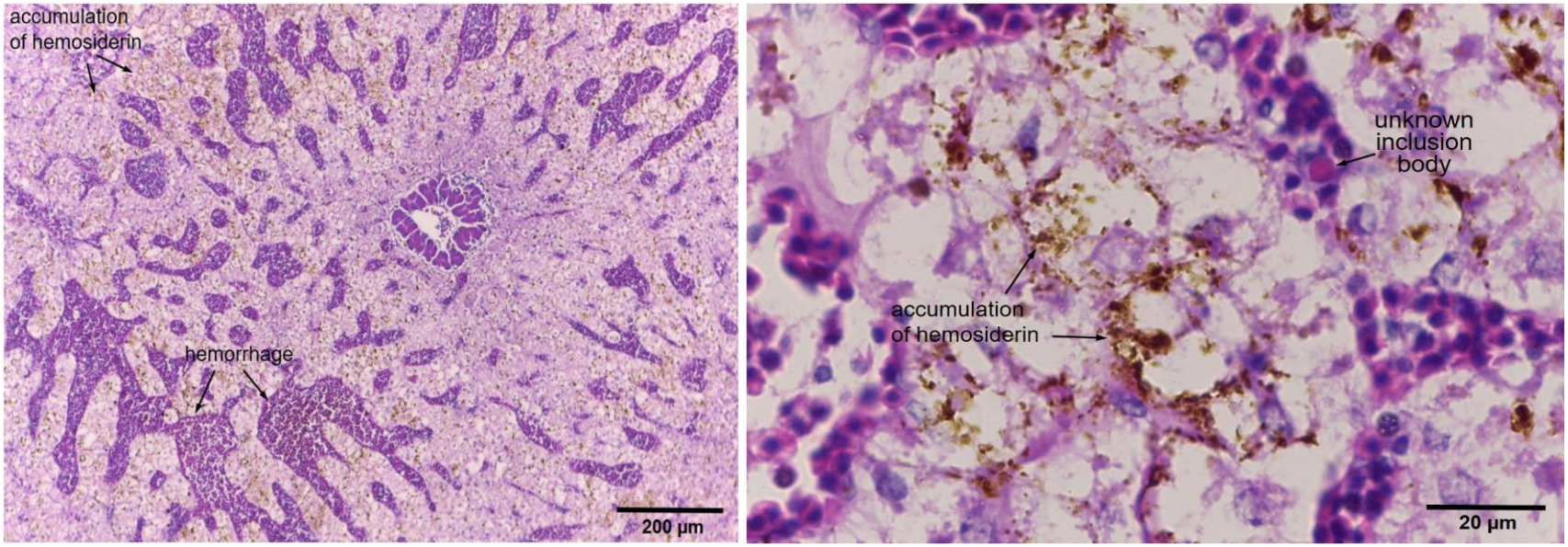
Liver of a hybrid red tilapia juvenile infected with *Edwardsiella ictaluri* showing severe blood congestion

Histopathology of other infections



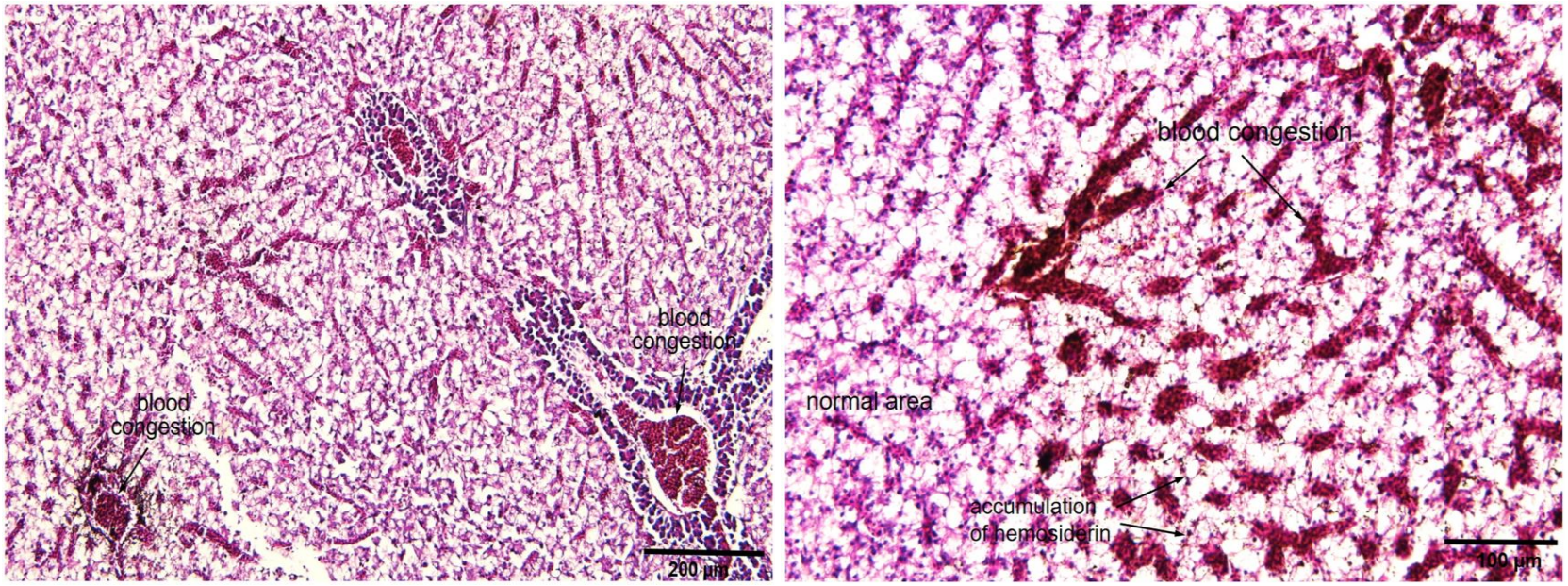
Photomicrographs of the H&E stained liver of an adult Nile tilapia infected with *Streptococcus agalactiae* showing hyperactivation of melanomacrophage centers with overloaded melanophores in exocrine pancreas (A), severe hepatocyte degeneration and accumulation of melanophores (B).

Histopathology of other infections



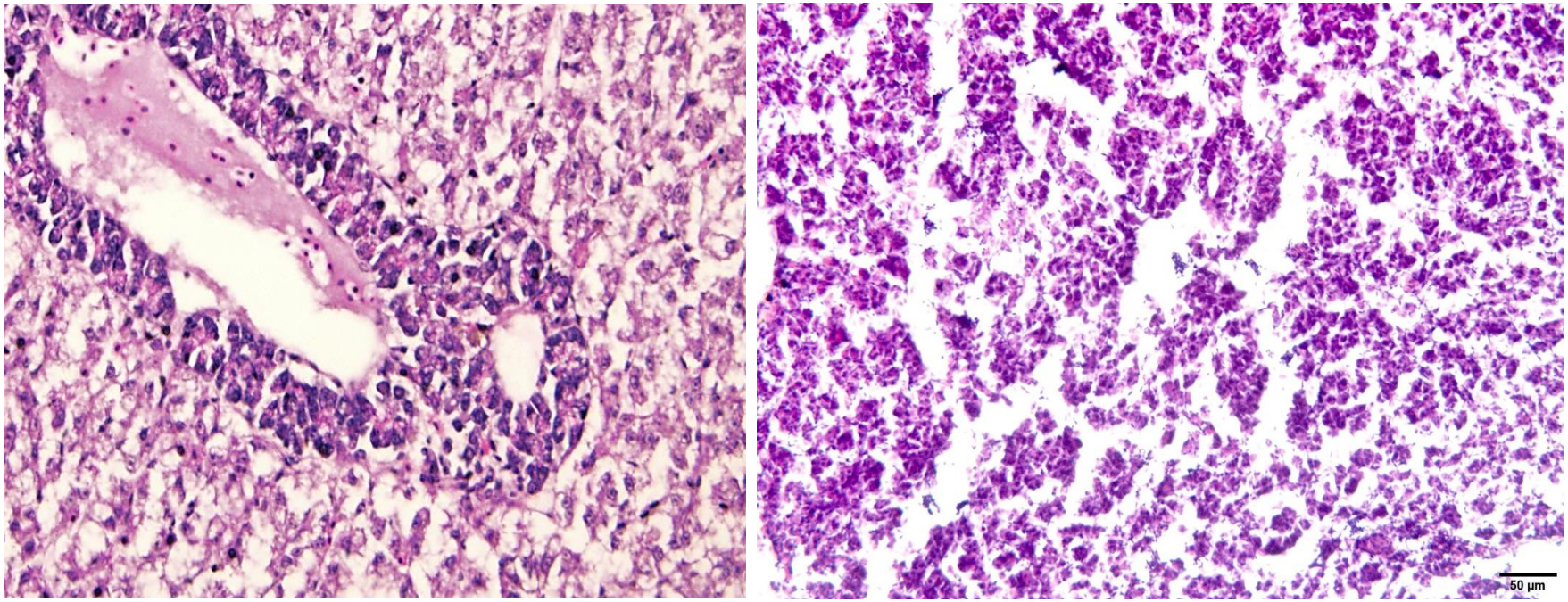
Photomicrographs of the H&E stained liver of a hybrid red tilapia sub-adult concurrently infected with *Streptococcus agalactiae* and *Micrococcus* sp. revealed severe hemorrhage, tissue degeneration and accumulation of hemosiderin.

Histopathology of other infections



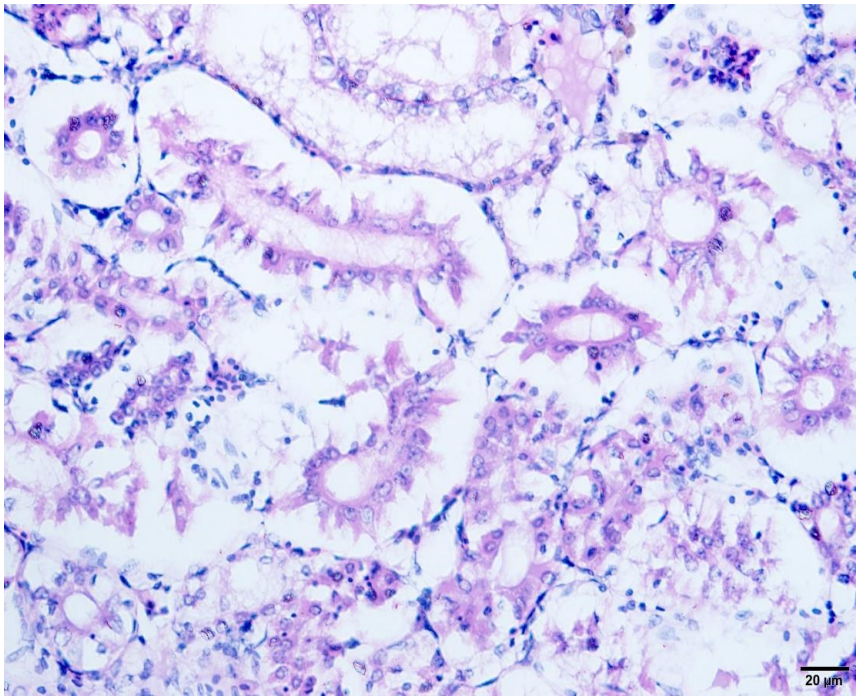
Photomicrographs of the H&E stained liver of an adult Nile tilapia infected with *Aeromonas veronii* showing severe blood congestion and accumulation of hemosiderin.

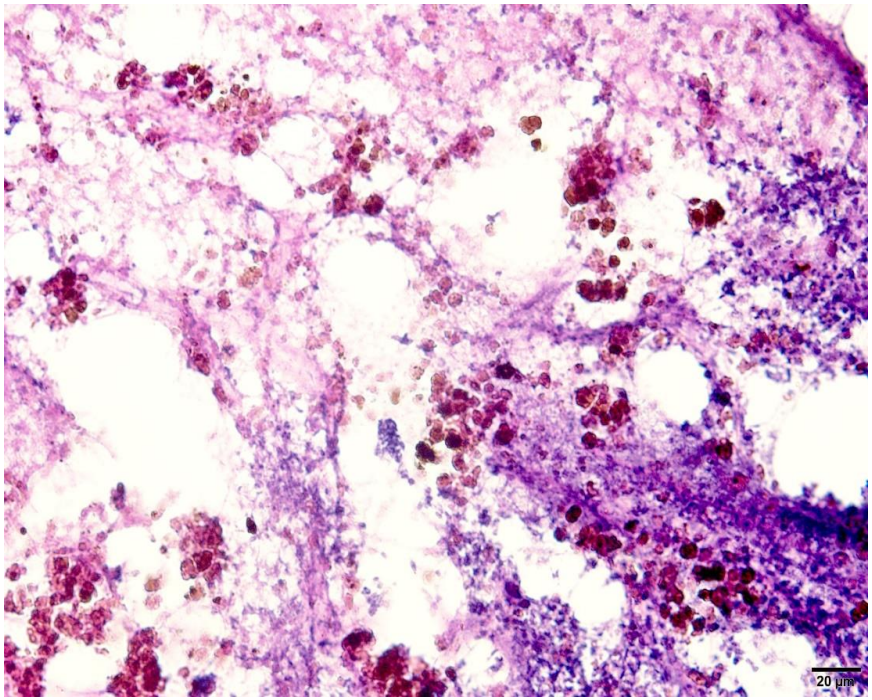
Tissue degeneration due to post mortem changes



H&E histology of liver showing post mortem changes

Tissue degeneration due to post mortem changes





H&E histology of kidney (left) and spleen (right) showing post mortem changes

In situ hybridization (ISH)

Purpose

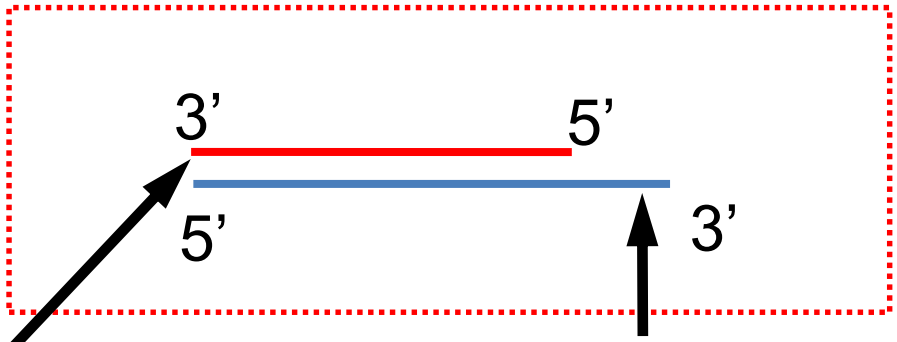
- To detect and confirm the presence of TiLV (through its nucleic acid) in the tissues and histopathological lesions
- To identify tissue tropisms of TiLV

In - situ hybridization

inside site

"in position" or "in place"

a hybrid molecule



TiLV specific probe

TiLV RNA in the cell

"Basically it involves formation of a hybrid molecule between an endogenous single-stranded RNA or DNA in the cell and a complementary single-stranded RNA or DNA probe"

Gall (2016) Methods 98:4-9

Development of TiLV-specific ISH probe

TiLV-infected tilapia

↓

Extraction of total RNA

↓

RT-PCR amplification of 415 bp genomic segment-3

↓

clone a TiLV genomic fragment into a plasmid

↓

PCR labeling with digoxigenin-11-dUTP

↓

Purified DIG-labeled TiLV probe

DNA probes preparation

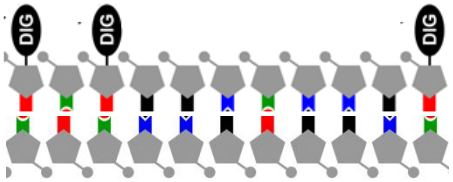
- Probes were prepared using DIG-labeling Mix (Roche, Germany)
- Plasmid pGEM-415 bp was used as a template in the labeling reaction
- 282-bp fragment derived from IMNV was employed as an unrelated negative probe

TATGCAGTACTTTCCCTGCCTGAGTTGTGCTTCTAGCAA
TCAACATCAAAAGCTCACGAGCAAGTGGGGCACTAGCTG
GTAGAGGCAATATCTTCTGTGTAGCAGGCTTATGAGAAG
CAACTGTATACCTTTGTATCCACCCTCCATTGCGGAACT
CAAATTCTC**TATCACGTGCGTACTCGTTCAGT**ATAAGCT
CTCTTGCCTCTTGGTCAAGACCACACTCCTCACCACAGG
CGAGGAACTTTGAGCACTCGAAGAACCCATATTGCCTCT
TTAGCTCAGCTGTCTCCTTGGATATGTCCGCGAGTCTGG
GTGGTGCCACCCACTCGATACGAGGCTTCGGGCCACTCT
TTGGATGTGGTAGTTCAAATAGCCGTTCCCTTAGCTCAG
CATCG**TAGGATGCCTTGTGCCCAAC**

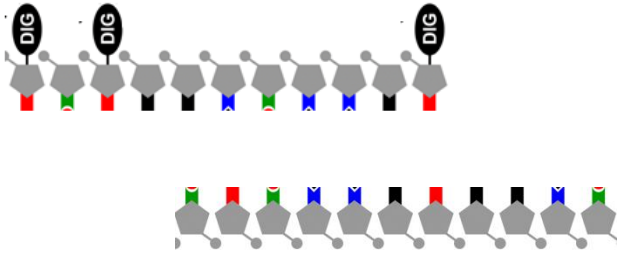
Sequence of a 415-bp derived from genome segment 3 of TiLV cloned in pGEM-T. (used primer Nested ext-1 & ME1)

Principle of *in situ* hybridization (ISH)

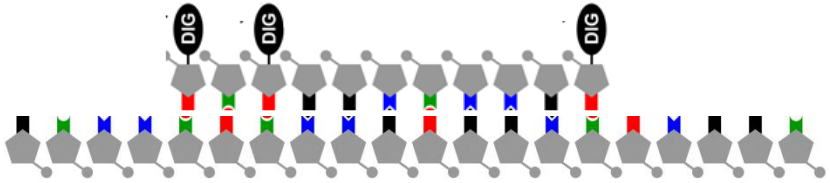
1. Labeling DNA probe



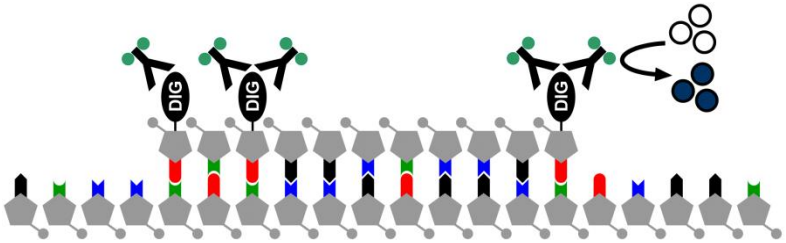
2. Denaturation of DNA probe



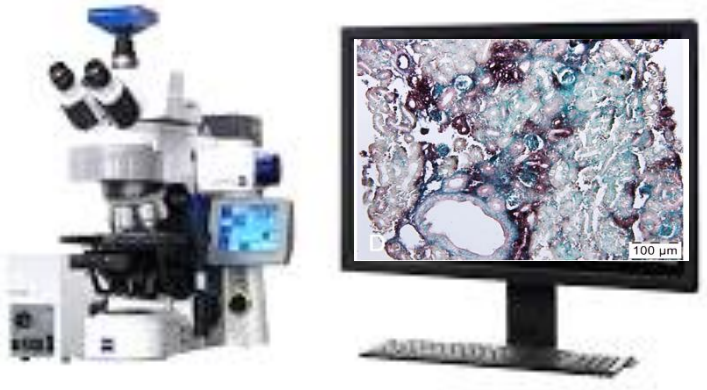
3. Hybridization

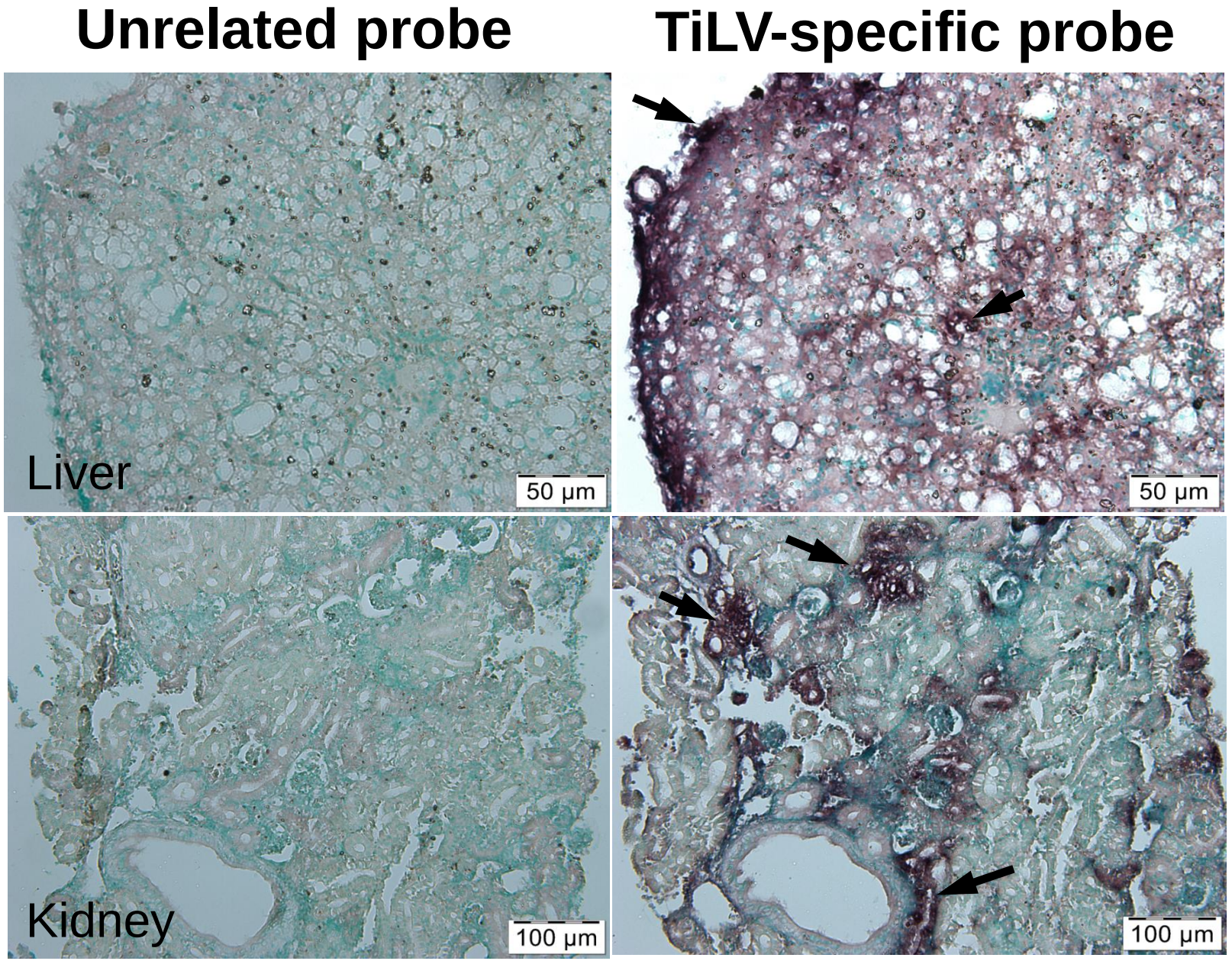


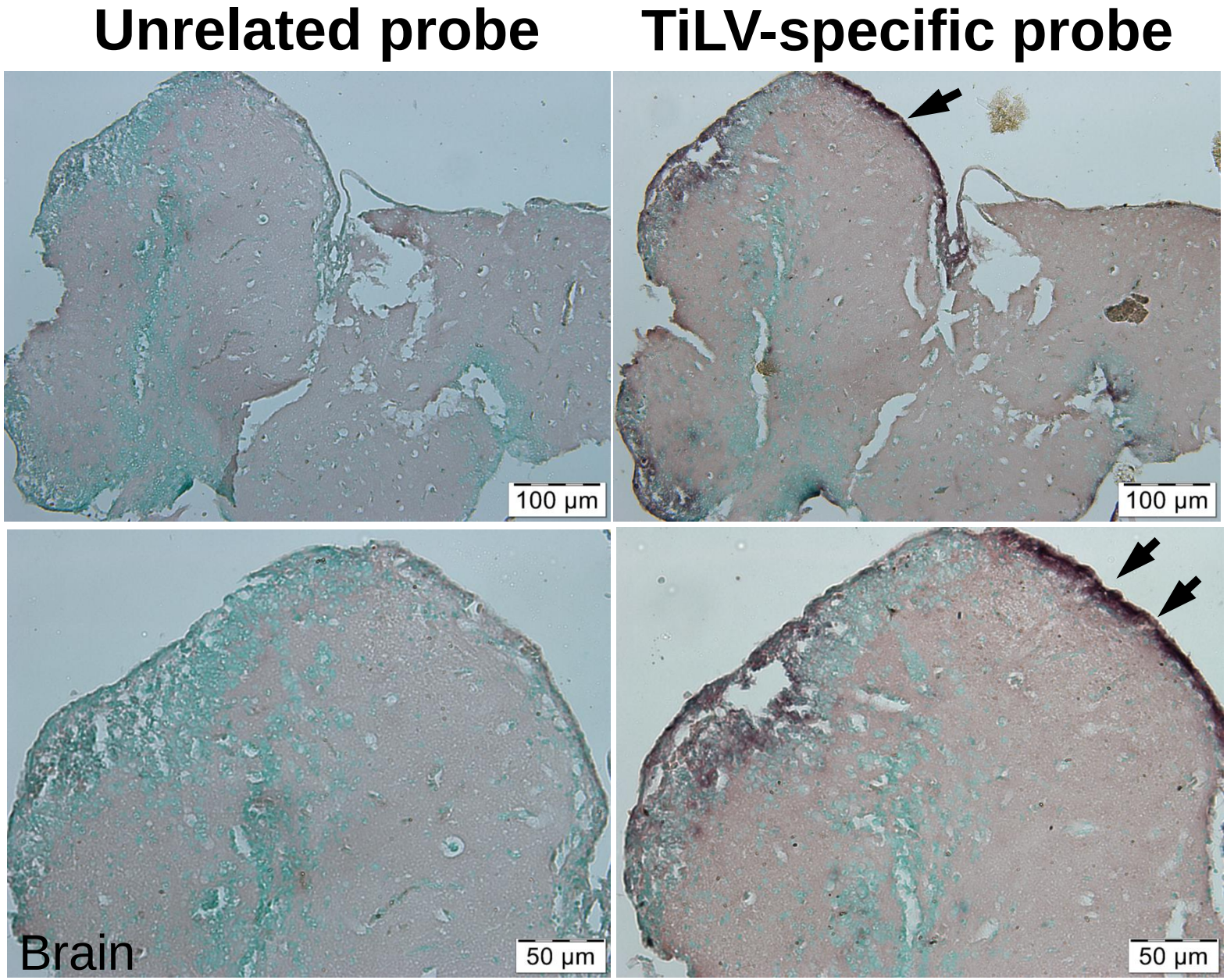
4. Color development

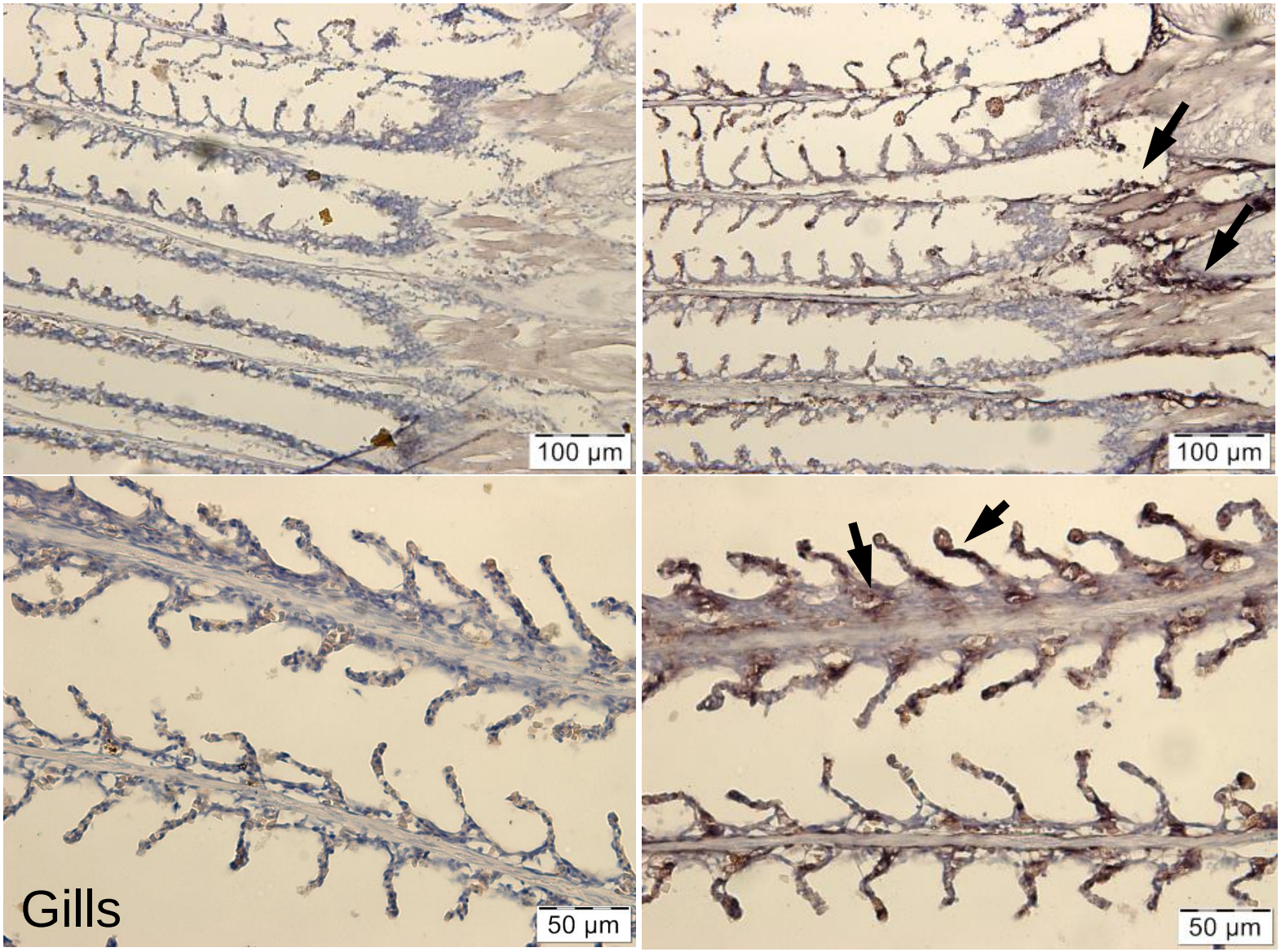


5. Result interpretation




Unrelated probe
TiLV-specific probe
Liver
50 µm
50 µm
Kidney
100 µm
100 µm


Unrelated probe
TiLV-specific probe
100 µm
100 µm
Brain
50 µm
50 µm


Unrelated probe
TiLV-specific probe
100 µm
100 µm
Gills
50 µm
50 µm

Sample collection for histology

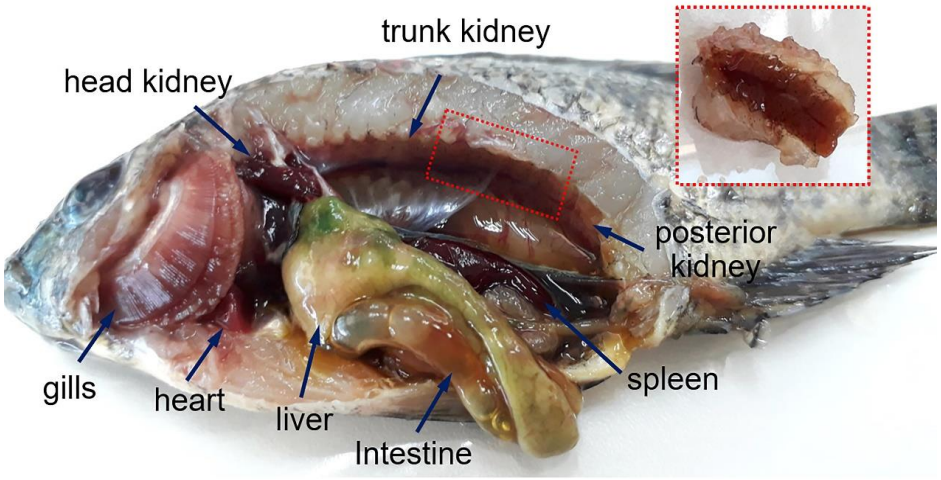
Sample collection for histology

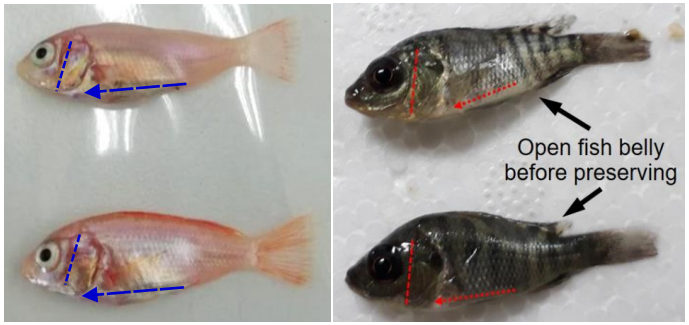
- Samples should be collected from moribund fish or freshly dead fish (best within 15 min post mortem)
- Do not use frozen fish
- Liver is the best tissue for TiLVD histopathological diagnosis
- Additional organs, such as kidney, spleen, brain, gills may be useful

Sample collection for histology

Samples

- **Fry and fingerlings can be preserved whole**
  - Remove gill opercula
  - Open fish cavity by a cut along midline and viscera should be pulled out to allow fixative to penetrate properly into the tissues
- **For bigger fish, necropsy should be performed**
  - Small pieces (~3-5 mm thickness) of individual organs should be collected and preserved in fixative





Sample collection for histology

Fixation

The purpose of tissue fixation is to permanently preserve the tissues in a life-like state and to prevent autolysis and decomposition.

10% neutral buffered formalin (NBF)

- 37% Formaldehyde: 50 mL
- Distilled water: 450 mL
- Sodium phosphate, diabasic (Na_2HPO_4): 3.25 gm
- Sodium phosphate, monobasic (NaH_2PO_2): 2 gm

Combine all ingredients and mix well, label and date. Store at room temperature

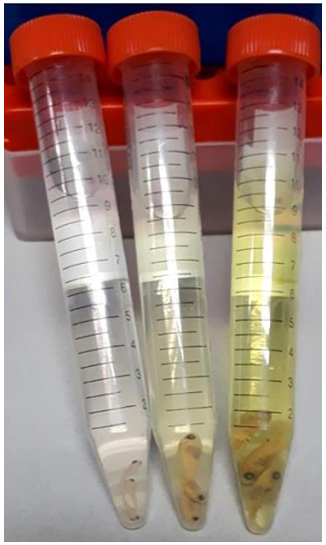
Bouin's Fixative

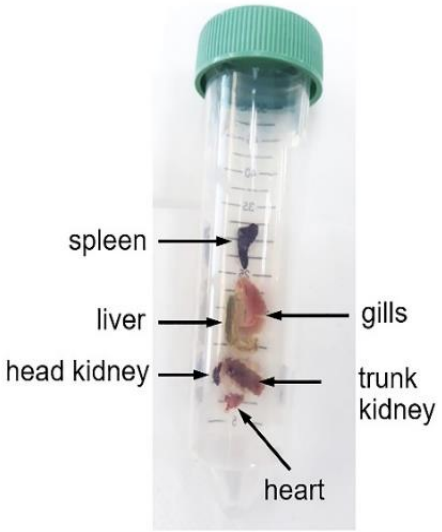
- Saturated picric acid: 3000 mL
- 37% Formaldehyde: 1000 mL
- Glacial acetic acid: 200 mL

Combine all ingredients and mix well, label and date. Store at room temperature

Sample collection for histology

- Ratio of sample: fixative should be 1:10 (w/v)
- After 12-24 hours, preserved tissues should be transferred to 70% ethanol (ratio 1:10 (w/v)) for long-term storage

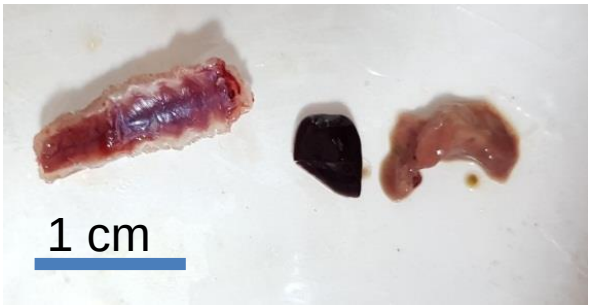




What should be avoided

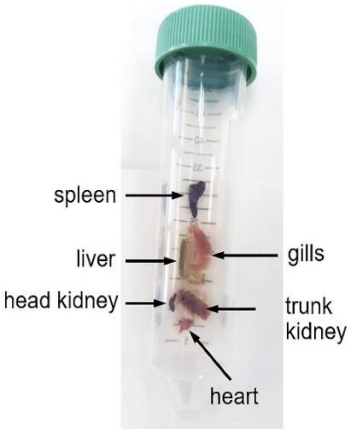
- Dead fish → post mortem change
- Dissection takes too long time → autolysis
- Contamination
- Physical destruction of tissue → not good for histology
- Tissue pieces are too big
- Fixative is not enough
- Sample in formalin 10% for too long → not good for ISH

Not enough fixative



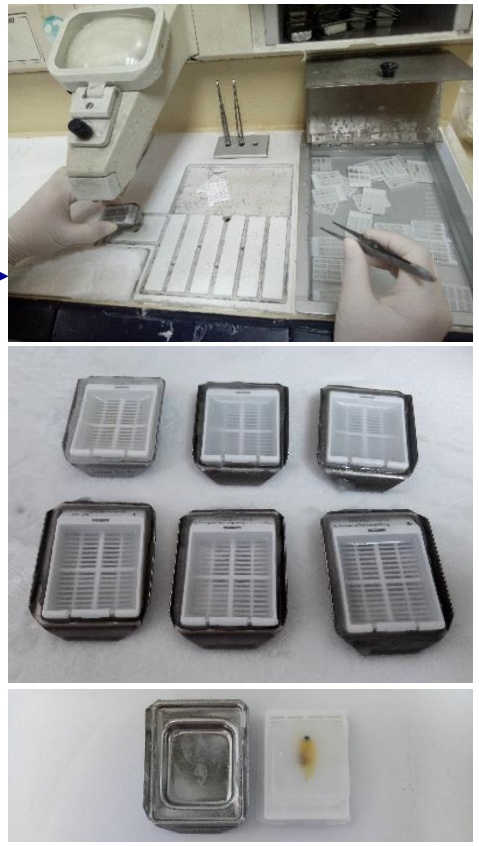
good size for preservation

Tissue processing & Embedding



Samples in alcohol 70%

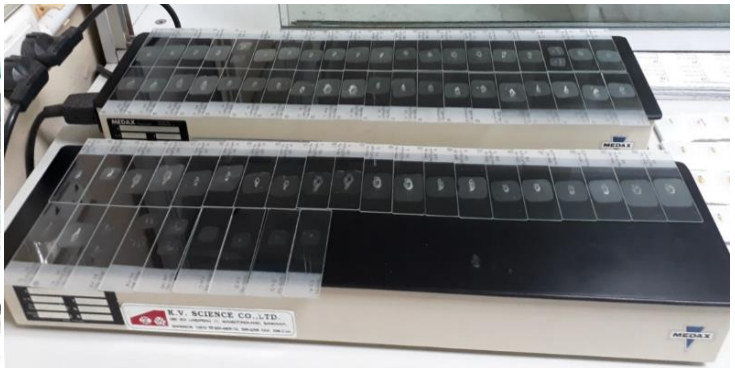
Dehydration of samples in an automatic tissue processing machine



Embed samples in molten paraffin

Sectioning


Section embedded tissue at 4-5 µm thickness

Float the tissue ribbon onto the surface of a ~45 °C water bath before placing sections onto slides

Hematoxylin and Eosin (H&E) staining

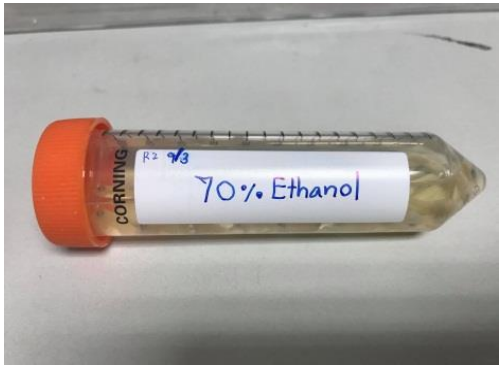
Staining of sections with hematoxylin and eosin (H&E)

Mounting slides, examination under a light microscope

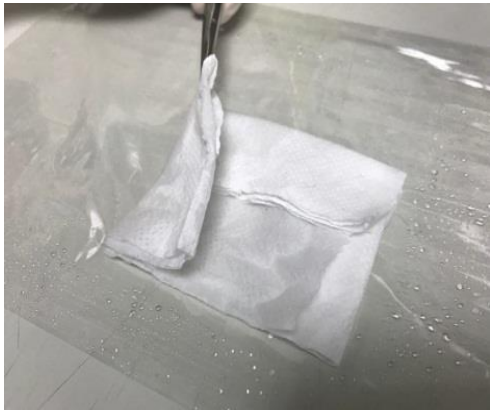
- **Hematoxylin (Blue/Purple)** stains basophilic substances in cells e.g. the nucleus and chromatin
- **Eosin (Pink)** stains all eosinophilic substances in cells not stained by hematoxylin e.g. cytoplasm, collagen, muscle fibers

Shipping histology samples

Shipping histology samples



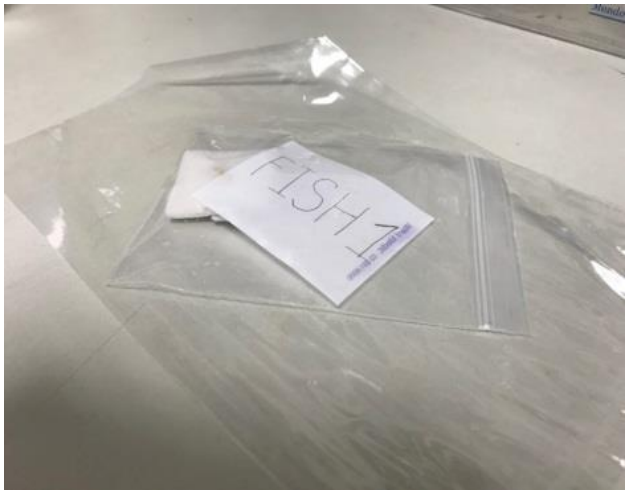
Sample in 70% ethanol



Wrap fixed sample (in 70% ethanol) with paper towel.

Shipping histology samples

Add 70% ethanol into bag to saturate the paper towel.



Label on sturdy paper/written in pencil and insert into the bag.

Place in second and third plastic zip lock bag.

Shipping histology samples

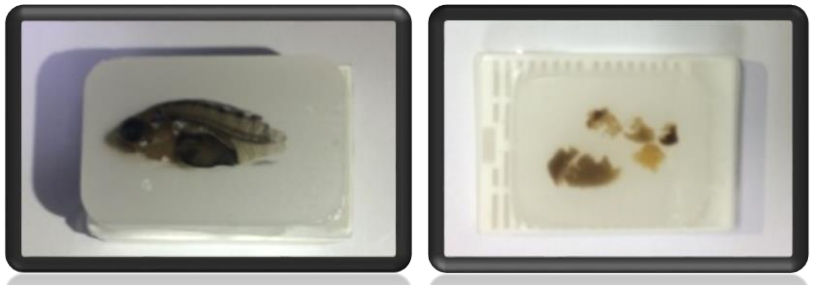
Place package into a sturdy container for shipping

Shipping histology samples

Important notes:

- Glass containers are not recommended due to the risk of breakage.
- Sending large volumes of fluid is costly and dangerous if the container becomes broken.
- Labels should be made of sturdy paper (e.g. plastic paper) and written with a pencil.
- Ink/marker will dissolve when exposed to ethanol.
- Always send an accompanying letter giving information regarding the sample: type of test, species, sample identification, history of sample, and a contact person for report and invoice.

Shipping histology samples



Paraffin blocks

H&E stained slides

- ✓ These forms of samples are convenient for shipping through normal post mail

Thank you for your kind attention