Système d'Informations Halieutiques

Action Paramètres biologiques

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Sample collection protocol for maturity staging of marine fish through histology

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1. Context

This protocol was set up under the MATO project (MATurité Objective des poissons par histologie quantitative), as part of Carine Sauger's PhD. The MATO project, financed by IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer), aims at improving the evaluation of sexual maturity stages of exploited stock species using histology paired with a stereology reading process.

The present document (version 1.0 as of June 2023) gathers protocols used in the study of marine female fish gonads as well as the study of the different cellular structures found in ovaries of the following species : plaice (*Pleuronectes platessa*)(Kellner & Sauger, 2019), megrim (*Lepidorhombus whiffiagonis*)(Heude-Berthelin et al., 2023b), four-spot megrim (*Lepidorhombus boscii*)(Heude-Berthelin et al., 2023a), red mullet (*Mullus surmuletus*)(Berthelin et al., 2023), and blue whiting (*Micromesistius poutassou*). Materials used here are not always the most optimal but favor a manual approach.

Most of the histological methods found in this protocol are based on methods described by Gabe, or were modified so as to answer our queries from a fisheries point of view, from the collection of fresh samples to the staining and mounting of histological slides, including inclusion in paraffin (Manfred, 1968; Martoja-Pierson & Martoja, 1967).

For the detailed histological slide reading method used, refer to the 'Stereology reading protocol' (Dubroca et al., 2023). For detailed descriptions of ovarian cellular structures for one of the species cited above, please refer to their corresponding lexicon.

We thank the BOREA Laboratory of University of Caen Normandy for the following protocols.

2. List of materials

2.1. Reagents

Refer to the appropriate material safety data sheet provided by the manufacturer. Be sure to handle chemical reagents under a fume hood and wear suitable personal protective equipment, including a laboratory coat, nitrile gloves and safety goggles.

- **Davidson's fixative** (see set up *Annex 4*). Davidson's fixative is flammable and toxic if inhaled, swallowed or absorbed through the skin.
- **Ethanol** (absolute, C_6H_6O, Mw=46.07) (CHIMIE-PLUS Laboratoires, ref.40079). For all ethanol dilutions, refer to the Gay-Lussac table (*Annex 3*). Ethanol is flammable.
- **Butanol** (absolute, $C_4H_{10}O$, Mw=74.12) (CHIMIE-PLUS Laboratoires, ref.24022). Butanol is flammable and toxic if inhaled, swallowed or absorbed through the skin.
- **Paraffin** (VWR, ref.2079-A, Klinipath). There will be presence of butanol in paraffin waste after dehydration.
- **Diasolv®** (Diapath, ref.X0016). Diasolv® is flammable and toxic if inhaled, swallowed or absorbed through the skin.
- **Fast Green (0.2°)** ($C_{37}H_{34}N_2Na_2O_9S_3$, Mw=792.86) (ROTH, ref.7706.3) (see set up *Annex 5*). Fast Green is dangerous for the environment.
- **Hematoxylin** ($C_{16}H_{14}O_6.nH_2O$, Mw=302.28) (Sigmaaldrich, ref.H3136) (see set up *Annex 5*). Hematoxylin is flammable.
- **Eosin Y (1°)** ($C_{20}H_6Br_4Na_2O_5$, Mw=691.85) (Eosin Y disodium salt, Sigmaaldrich, ref.E4382) (see set up *Annex 5*). Eosin is dangerous for the environment.
- **Phosphomolybdic acid (1°)** ($H_3[P(Mo_3O_{10})_4].nH_2O$, Mw=1825.25)(VWR, ref.20616.138) (see set up *Annex 5*).
- Distilled water (dH_2O)
- **Diamount®** (Diapath, ref.030400). Diamount® is toxic if inhaled, swallowed or absorbed through the skin.
- **Glycerol** ($C_3H_8O_3$, Mw=92.09) (86°, ROTH, ref.7533.3)
- **Formaldehyde** (CH_2O, Mw=30.03) (37°, ROTH, ref.7398.5)
- **Filtered sea water**
- **Acetic acid** ($C_2H_4O_2$, Mw=60.05) (VWR, ref.20103.295)
- **Sulphuric acid** (H_2SO_4, Mw=98.08) (95°, VWR, ref.20700.298)
- **Ammonium Iron (II) sulphate hexahydrate** ($H_8FeN_2O_8S_2.6H_2O$, Mw=392.13) (VWR, ref.24257.260)

2.2. Equipment

- Dissection kit (scalpel, surgical scissors, flat forceps, thin forceps, dissection knife) (*Fig. 1*)
- Thin tip permanent marker, pencil
- Groove director
- Long stainless steel forceps (30cm)
- Camera (We used Olympus TOUGH F2.0 model)
- Dissection board
- Graduated ruler (cm)

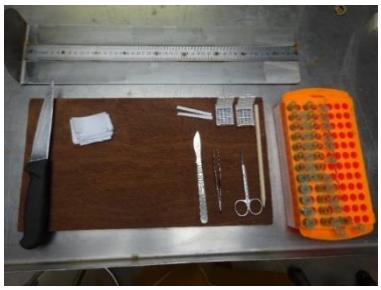


Figure 1. Dissection kit

- Tissue processing embedding cassettes (Simport, ref.M512)
- Eppendorf (VWR, ref.525-1134) or envelope for otoliths
- Presicion scale (>1kg, presicion 0.01g)
- Scale (max5kg, presicion 1g)
- Petri dish (or small container to hold the gonads)
- Tags for the tissue processing embedding cassettes, made with conventional paper (0.5x5cm) (*Annex 1*)
- Identification tag for fish
- Data entry sheet to collect data (*Annex 2*)
- Refrigerator (4-1°C)
- Fume hood
- Plastic sample bottle with seal (2L) (*Fig. 5*)
- Measuring cylinder (1000mL)
- Laboratory waste bin for needle, blades and glass slides
- Chemical waste bin
- Paper towel
- Silicon mould (24 mould, 14x2.6x1cm, Blumtal) (*Fig. 2*)

Figure 2. Silicon moulds used to embed samples into paraffin blocs

- Proofing over (60°C max)
- Stainless steel beaker with spout + handle (500mL) (BOCHEM, ref.8501)
- 5 resealable containers for bath (wide neck bottle)
- Microtome (Leica, ref.14045746960)
- Microtome razor blades (Microm Microtech35+, ref.201219-74)
- Water bath (55°C with reverse osmosis treated water)
- Strait tip teasing needle
- Glass microscope slides (GHÄASEL, ref.BPB036)
- Brush with soft bristles

- Microscope
- Ice
- Timers
- Filters
- Funnel (DUTSCHER, ref.PNB013)
- Spoon/micro-spatula
- 16 plastic tanks for slide staining (Simport, ref.M900-12B)
- Slide staining holder (Simport, ref.M900-12B) (*Fig. 3*)

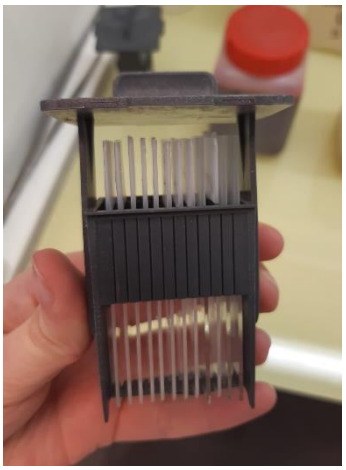


Figure 3. Slides staining holder

- 8 brown glass bottles or aluminium-covered vials to store products in the dark
- Disposable pipettes (10mL, EPPENDORF, ref. 0030127722)
- Coverslips (2.4x5cm, Biosigma, ref.VB5650)
- Disposable dropper pipettes (VWR, ref.612-4494)
- Plastic plate
- Scanner (We used VS120 Olympus model driven by Olympus VS-ASW software)
- Cardboard storage box (*Fig. 4*)

Figure 4. Cardboard storage box.

Figure 5. Plastic sample bottle with seal

3. Sampling and fixation

3.1. Macroscopic parameters to collect on the field

The data to collect for each fish in the data entry sheet (*Annex 2*) is as follow:

- **ID Fish** (IFREMER : **ID Otolith**)
- **Species name** (IFREMER : codification index Rubin type)
- **ICES division** (ICES division where the fish was caught)
- **ICES Stat Rectangle** (ICES fisheries statistical rectangle where the fish was caught)
- **Date** (DD/MM/YYYY)
- **Total length of the fish** (precision to the 1cm or ½cm, rounded down)
- **Ungutted fish weight** (precision of 1g, rounded down)
- **Parasites presence** (Presence : Y or Absence : N)
- **Age**
- **Visual maturity** (ICES WKASMSF scale, 2018)
- **Liver weight in g** (precision of 0.01g rounded down)
- **Dorsal/Right gonad weight** (precision of 0.01g, rounded down)
- **Ventral/Left gonad weight** (precision of 0.01g, rounded down)
- **Number of sections**
- **Vessel** (IFREMER : Name of the vessel that carried out the fishing effort)

The **Total length of the fish** and the **Ungutted fish weight** must be collected following the methods recommended by the **associated campaign protocol** (IFREMER : Guides d'identification SIH (Garren, 2020)).

The **ID Otolith** is a unique identifier for each individual. It is generated by Imagine (Elleboode et al., 2022). This identifier will follow the sample throughout its processing and the associated information will be incorporated into the Imagine database, as recommended by IFREMER.

The **Number of sections** is the number of gonad sections extracted for each individual. For example, if, for a specific individual, only one section was sampled for each ovary, there are therefore two samples for this individual. Note down "2" in this column for this fish.

The **Visual maturity** is estimated following the macroscopic criteria from the WKASMSF scale (ICES, 2018) (*Table 1*). This European scale with six maturity phases is a universal scale for every species of bony fish. Each maturity phase is associated with predefined visual criteria (gonad appearance, content, colour…) (ICES, 2012, 2018). For species identified during IFREMER campaigns, sexual maturity phase identification sheets were set up (Le Meleder & Dubroca, 2022).

Table 1. Table adapted from the ICES maturity scale « WKASMSF » (ICES, 2018).

Status	Code	Maturity phases
Sexually immature	A	IMMATURE
Sexually mature	B	DEVELOPING
	C	SPAWNING
	D	REGRESSION/REGENERATION
	E	OMITTED SPAWNING
	F	ABNORMAL

3.2. Tag for tissue processing embedding cassettes

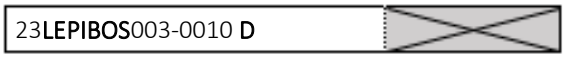
For each sample, an identification tag (*Annex 1*), made with conventional paper and standardized in size to the embedding cassettes, is placed folded in half in the embedding cassettes. The ID Otolith on the tag is generated by Imagine (Elleboode et al., 2022) and can be written on the tag with a pencil or with printer ink.

On each tag, write down the **ID Otolith** + **Sampled gonad position**

- Flat fish tag :
  - Ventral gonad = **ID Otolith** + **V**
  - Dorsal gonad = **ID Otolith** + **D**
- Round fish tag :
  - Left gonad = **ID Otolith** + **G**
  - Right gonad = **ID Otolith** + **D**

Do not write anything on the grey section!
This part will be included into the paraffin bloc

Tag example :



3.3. Gonad's photography

Several photos are to be taken for each individual. These photos must include the identification tag of the individual, as well as its sex and its maturity phase. These pictures are added to the dataset "**A gonad photographs dataset for fish of commercial interest**" (Le Meleder *et al.*, 2022), and are used to complete the maturity identification guide for fish of commercial interest (https://lm-anna.github.io/MaturityScaleTools/) (Le Meleder & Dubroca, 2022).

Here is the list of all the different types of photos to take:

- **Photos of the whole fish on a graduated ruler**
- **Photos of the gonads inside the abdominal cavity of the fish** (with or without the other organs depending on the state of the fish) (*Fig. 6ab*)
- **Photos of the gonads outside of the abdominal cavity of the fish** (*Fig. 6c*)

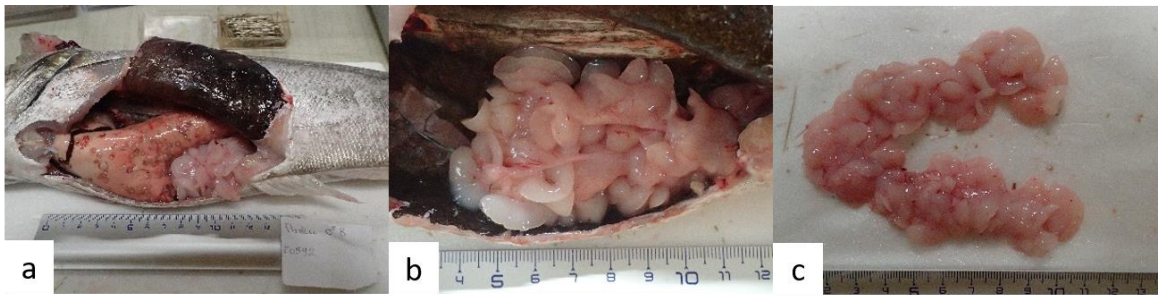


Figure 6. Different types of photos: a) Gonads still inside abdominal cavity of the fish with other organs; b) gonads inside the fish without other organs; c) gonads outside the fish

The photos should be taken on a white background, with a graduated ruler and in a well-lit environment. Moreover, the **ID Fish** must be visible. Finally, for the photos of the gonads outside of the abdominal cavity, always place the dorsal/left gonad at the top and the ventral/right gonad at the bottom.

For further details about the methods used to photograph fish gonads, see the "**Fish gonad's photography protocol**" (Le Meleder, Sauger, & Dubroca, 2022).

3.4. Flat fish gonad extraction

- **Weight** (to the 1g rounded down) with a scale and **measure** (to the 1cm rounded down) the total length of the fish.
- **Collect the otoliths** and put them in an envelope/eppendorf with the ID Otolith of the fish.
- Place the fish on its ventral side, make a diagonal incision passing below the caudal fin, then parallel to the lateral line. Unfold the flesh to **bring into view the dorsal gonad without cutting it**, as shown below (Le Meleder et al., 2022).
- **Estimate the maturity phase** by following the WKASMSF scale criteria (ICES, 2018).
- **Take a photo** of the whole fish with the gonad inside the abdominal cavity on a white background with a graduated ruler and the **ID Otolith** (*Fig. 7*).
- **Gently extract the first gonad.** For female individuals, you must cut the ovaries at the base of the oviduct, where the two ovaries are connected together, in order to get them out of the abdominal cavity. **The gonad extracted from the back side of the flat fish is the dorsal gonad (D). The one extracted from the bottom side is the ventral gonad (V).**
- Turn the fish on its back and repeat the previous incisions to **extract the ventral gonad.**
- Take a photo of the two extracted gonads outside of the abdominal cavity on a white background, with a graduated ruler and the **ID Otolith** (*Fig. 8*). If possible, separate the gonads from one another and **place the dorsal gonad above the ventral one**.

Figure 8. Photo of a megrim on a white background, a ruler and its ID Otolith.

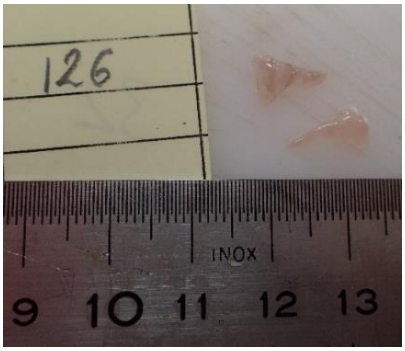


Figure 7. Photo of the two gonads outside the abdominal cavity, on a white background with a graduated ruler and their ID Otolith.

- **Weight** separately **each gonad** on a precision scale (0.01g rounded down).
- **Check the presence of parasites** inside and outside the fish.
- **Extract the liver and weight it** on the precision scale (0.01g rounded down).
- If the gonads are more than 3 cm long**, make a 2 cm section in the median area of the gonad.** Be careful not to take a section where the gonadal wall is torn or pierced.
- Place **a single gonad section in a flat position with its identification tag** (*Annex 1*) into a tissue processing embedding cassette. Avoid having the sample and the paper tag touching one another.
- Close the embedding cassettes and, under a fume hood with nitrile gloves, **submerge the embedding cassettes into Davidson's fixative for 24 to 48h** and place the bottle in the refrigerator (at least 48h for large samples with hydrated oocytes).

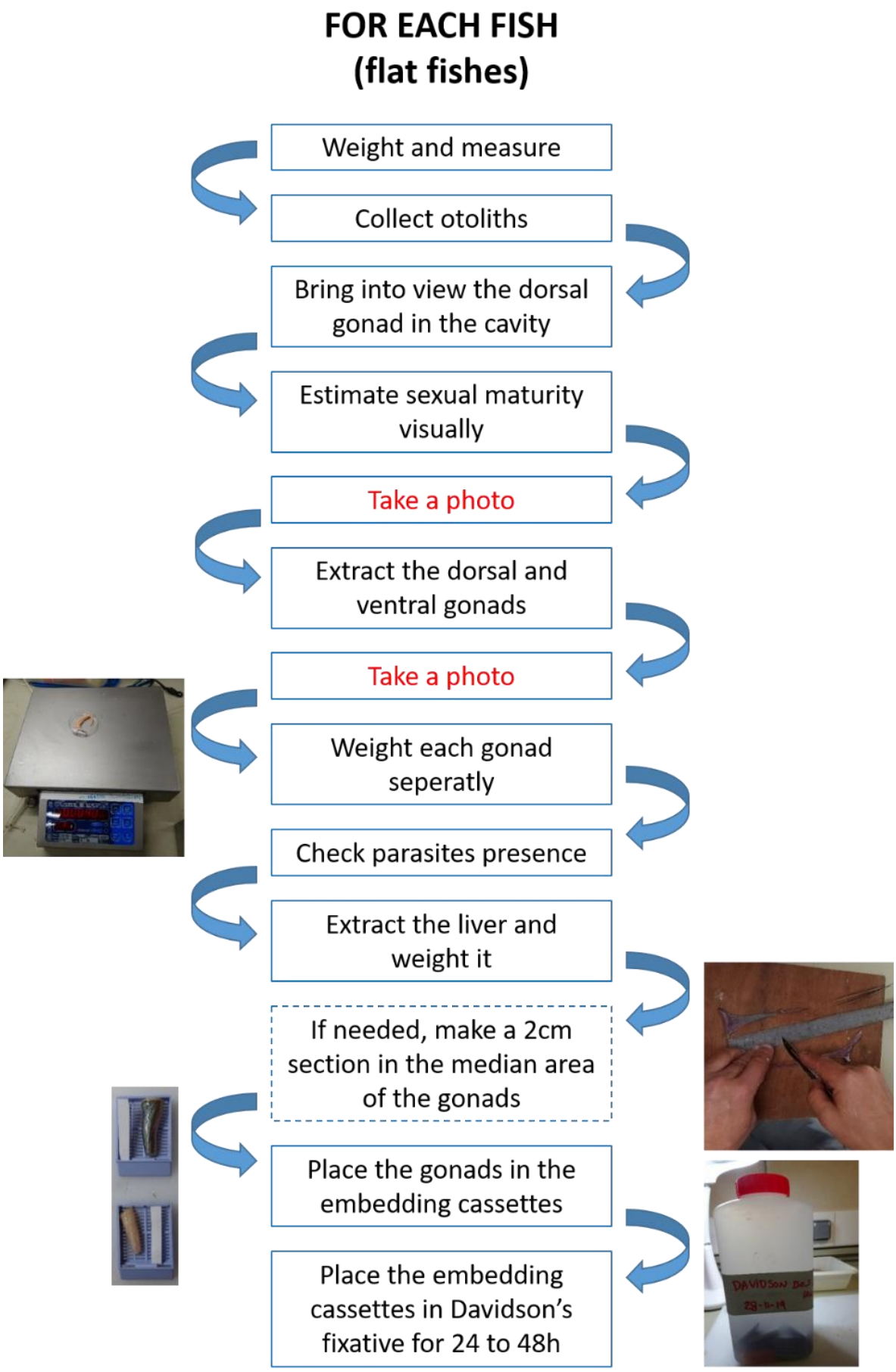


Figure 9. Diagram of the steps for gonad extraction of flat fishes.

3.5. Round fish gonad extraction

- **Weight** (to the 1g rounded down) with a scale and **measure** (to the 1cm rounded down) the total length of the fish.
- **Collect the otoliths** and put them in an envelope/Eppendorf, with the ID Otolith of the fish.
- Place the fish on its back, **make an** incision from the genital opening to the pelvic fins. To not damage the gonads, it is strongly advised to use a **groove director**. Then, **make four diagonal incisions** as shown below (*Fig. 10*).

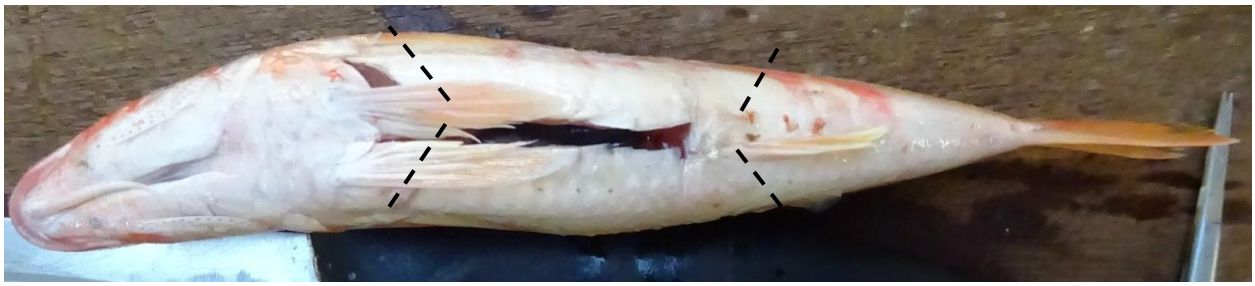


Figure 10. Incisions to open the abdominal cavity of round fishes.

- Unfold the flesh to **bring into view the dorsal gonads without cutting them**, and take a photo of the whole fish with the gonads in the abdominal cavity, on a white background, with a graduated ruler and the **ID Otolith** (*Fig. 11*) (Le Meleder et al., 2022).
- **Estimate the maturity phase** by following the WKASMSF scale criteria (ICES, 2018).
- **Gently extract both gonads** by cutting them at the base of the oviduct**,** where the two ovaries are connected to the genital opening, in order to get them out of the abdominal cavity. **When the fish is placed with its head to the left and its tail to the right, the uppermost gonad is the left one (G), and the lowermost gonad is the right one (D)** (*Fig. 12*)**.**
- **Take a photo** of the two extracted gonads outside of the abdominal cavity (*Fig. 12*)**.**

Figure 11. Opening of the abdominal cavity with a view on the gonads.

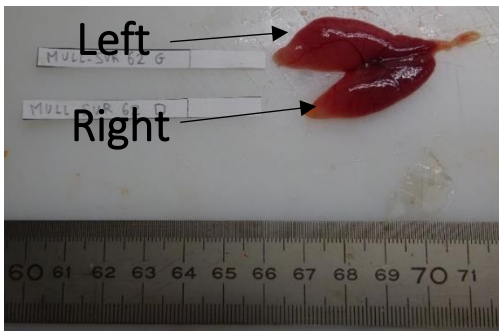


Figure 12. Photo of the gonads outside of the fish.

- For female fish, ovaries are often fused around their median or anterior area. This implies that there is a high risk of tearing the gonadal wall when separating them.
- **Separate gonads** and **weight them individually** on a precision scale 0.01g rounded down.
- **Check the presence of parasites** inside and outside of the fish.
- **Extract the liver and weight it** on a precision scale (0.01g rounded down).

- If the gonads are more than 3 cm long, **make a 2 cm section in the median area of the gonad.** Be careful not to take a section where the gonadal wall is torn or pierced.
- Place **a single gonad section in a flat position with its identification tag** (*Annex 1*) into a tissue processing embedding cassette. Avoid having the sample and the paper tag touching one another.
- Close the embedding cassettes and, under a fume hood with nitrile gloves, **submerge the embedding cassettes into Davidson's fixative for 24 to 48h** and place the bottle in the refrigerator (at least 48h for large samples with hydrated oocytes).

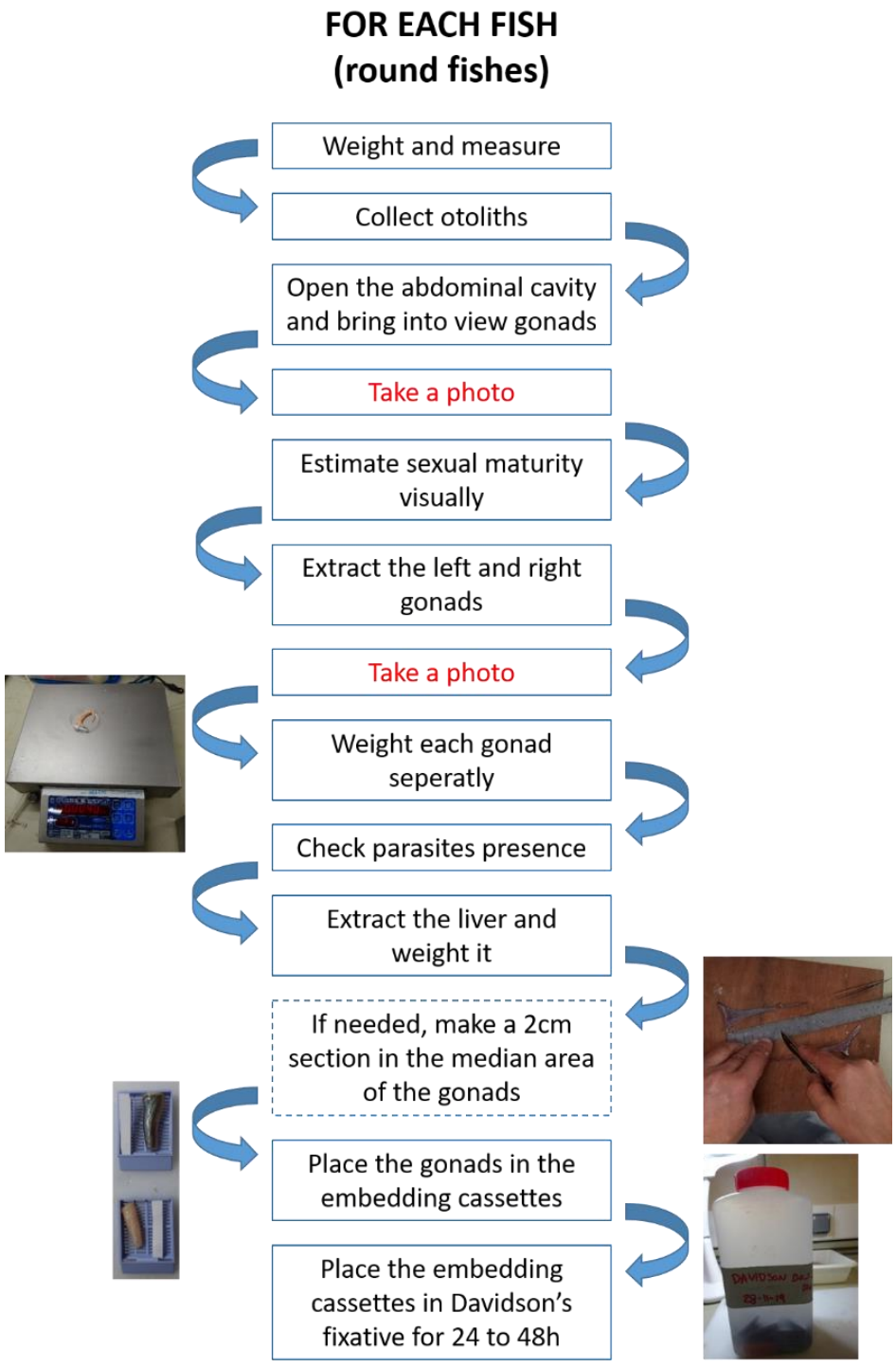


Figure 13. Diagram of the steps for gonad extraction of round fishes.

3.6. Cellular homogeneity

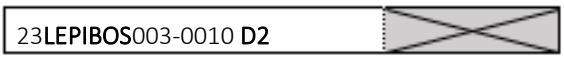
For marine fish, when monitoring a species for the first time, it is necessary to test the gonad's cellular homogeneity. This study is conducted to control if there are differences in cellular structures depending on location of the gonadal cross section is.

This test is carried out :

- only once per species and per sex
- on 15 individuals of identical maturity phases (preferably same sized fish, fished at the same time)
- not on immature individuals
- on more than 4cm long gonads
- avoid spawning individuals

The sampling methodology is similar to the one presented in section 3.4 and 3.5, the only difference being when the gonads are cut and placed into embedding cassette, after having being weighted separately.

- Place the dorsal/left gonad topmost and the ventral/right one below, while respecting the natural orientation the gonads were in when inside the fish's abdominal cavity. The tip of the gonad that was the closest to the fish's head must be on the left; it is the anterior part of the gonad. The tip of the gonad that was the closest to the fish's tail must be on the right; it is the posterior part of the gonad.
- Cut the gonads into three sections of at least 1 cm (2cm max): one section in the anterior area, one in the median area and one in the posterior area. Be careful not to tear the gonadal wall horizontally!
- In each embedding cassettes, put an identification tag (*Annex 1*) with the ID Otolith + sampled gonad position (V/G or D/D) + section position. Section position: 1 = Anterior, 2 = Median, 3 = Posterior (*Fig. 14*).
- Tag example for median section of a dorsal gonad (D2) sample from a four-spot megrim (LEPIBOS) :



- Place a single gonadal section in a flat position with its identification tag (*Annex 1*) into a tissue processing embedding cassette. Avoid having the sample and the paper tag touching one another.
- Close the embedding cassettes and, under a fume hood with nitrile gloves, submerge the embedding cassettes into Davidson's fixative for 24 to 48h and place the bottle in the refrigerator (at least 48h for large samples with hydrated oocytes).

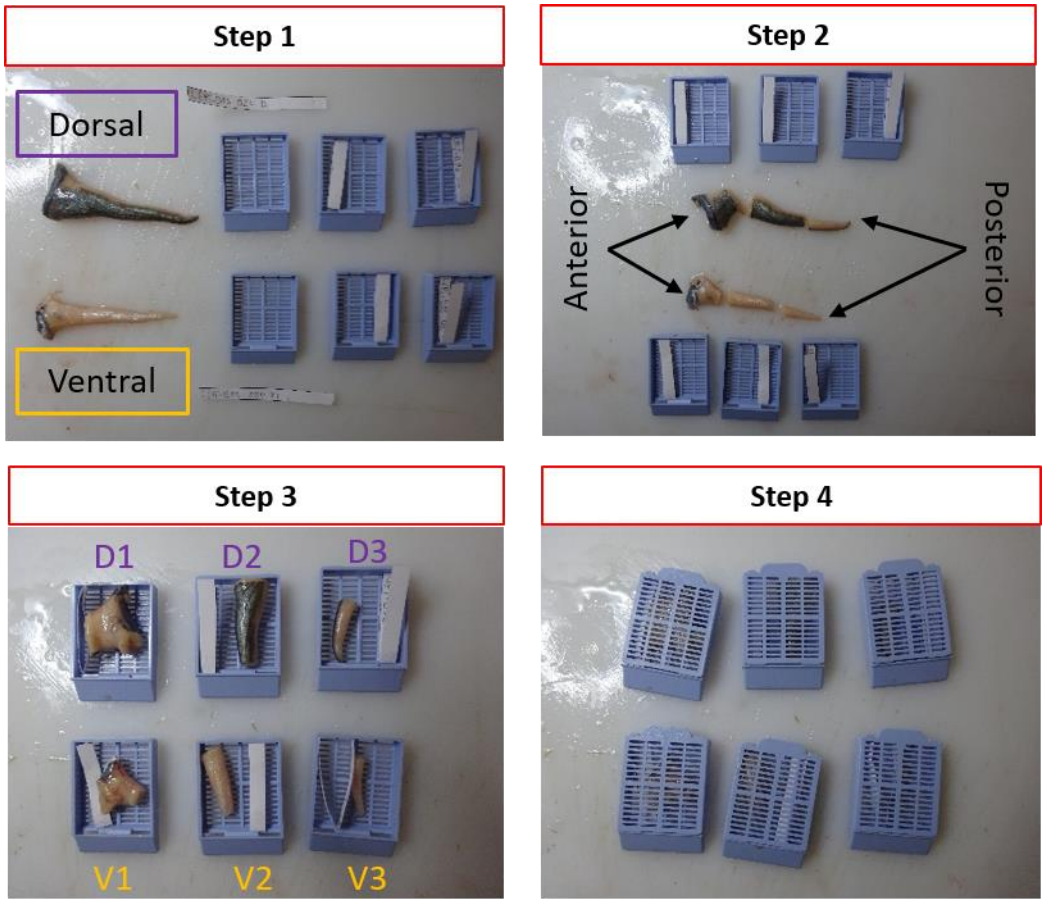


Figure 14. Diagram of the steps to sample gonads for homogeneity validation.

4. Sample trimming and conservation

After staying in **Davidson's fixative for 24 to 48h at 4°C**, samples will take a rubbery texture and **must be placed into 70° ethanol**.

Under a **fume hood and with nitrile gloves**, use long forceps to remove the embedding cassettes from Davidson's fixative and place them on paper towel (*Fig. 15*). The paper towel will absorb the overflow of fixative.

If Davidson's fixative is turbid (used for more than 120 samples and/or for ovaries rich in hydrated oocytes), empty it into the appropriate chemical waste bin using a funnel and retrieve the embedding cassettes.

For each embedding cassettes, **check if the identification tag is not stuck to the sample**. If that is the case, there is a high risk that the tag will tear up when being detached from the sample. In that case, a new tag must be made.

Next, **trim the edges of each sample** in order to have a straight edge (*Fig. 16*). This will make the following steps easier.

Finally, place the sample and tag back into their embedding cassettes, shut the cassettes firmly and place them in **70° ethanol**. Samples can then be stored in 70° ethanol for several weeks or months, at an ambient temperature if needed.

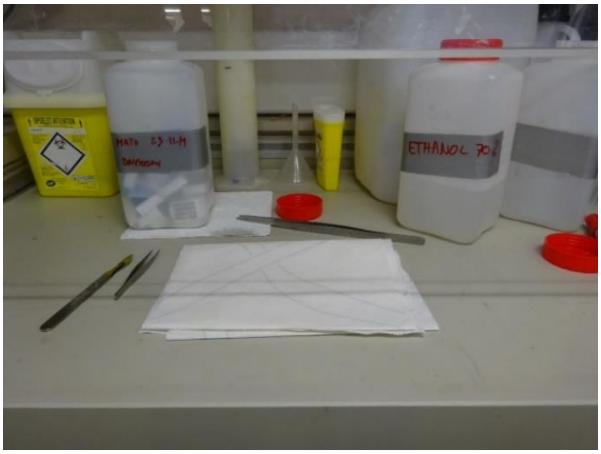


Figure 15. Materials to prepare under the fume hood.

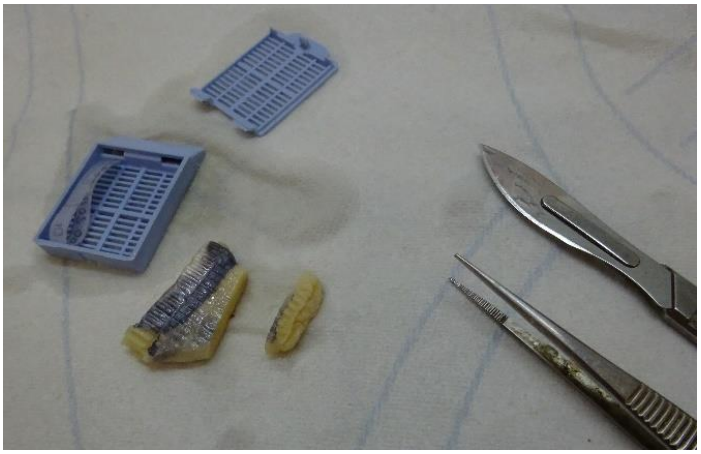


Figure 16. Trimming of the gonad's edges

Wastes :

- Davidson's fixative and ethanol are to be disposed in a can A 1993 FLAMMABLE LIQUID + CMR label

5. Dehydration and paraffin embedding

5.1. Manual dehydration

Following the trimming of the gonad's edges, samples will spend 24 to 48h (several weeks if needed) in 70° ethanol.

Before the manual dehydration, **do not forget to melt 500mL of paraffin in advance (at least 24h before) into two large beakers in the proofer at 60°C** (temperature of fusion will depend on the paraffin used). The volume of paraffin used must be completely submerge the volume of samples that need embedding by 2 cm. Change paraffin baths after every use. **Cover the beakers filled with paraffin to prevent butanol vapours from dispersing into the proofer.**

- Handle under fume hood with nitrile gloves and protective clothing.
- Avoid inhalation of toxic products (Davidson's fixative & butanol). 
- Use long stainless steel forceps to move samples from one bottle to another.

For a volume of 500mL and 15 samples of 2 cm, change ehtanol and butanol baths after 3 batches of samples, or if the alcohols turns turbid.

With long forceps, place the embedding cassettes into the following baths (*Fig. 17*):

1- 70° ethanol for 24 to 48h (or several weeks if needed)
2- 95° ethanol for 24 to 48h
3- 100° ethanol for 24 to 48h
4- 100° butanol for 24 to 48h (or several weeks if needed)
5- First liquid paraffin bath for 12 to 24h in 60°C proofer
6- Second liquid paraffin bath for 12 to 24h in 60°C proofer
7- Embed samples into paraffin (*Section 5.2*)

Wastes :

- Davidson's fixative, ethanol and butanol are to be disposed in a can A 1993 FLAMMABLE LIQUID + CMR label
- After solidification by cooling, paraffin is to be disposed of in suitable containers (presence of butanol in paraffin baths) A 2926 FLAMMABLE ORGANIC SOLID, TOXIC

Paraffin cleaning :

- **On surfaces**: use a putty knife to remove the bulk of dried paraffin. Clean with a sponge with hot water and liquid soap. Finish with a paper towel dabbed in 70° ethanol.
- **For stainless steel tools** (forceps…): wrap them in a paper towel and leave them in the proofer for a night at around 65°C.
- **Empty embedding cassettes (+ beakers):** place the empty embedding cassettes in a large beaker in the proofer for a night at around 65°C, then clean them under hot water with liquid soap.

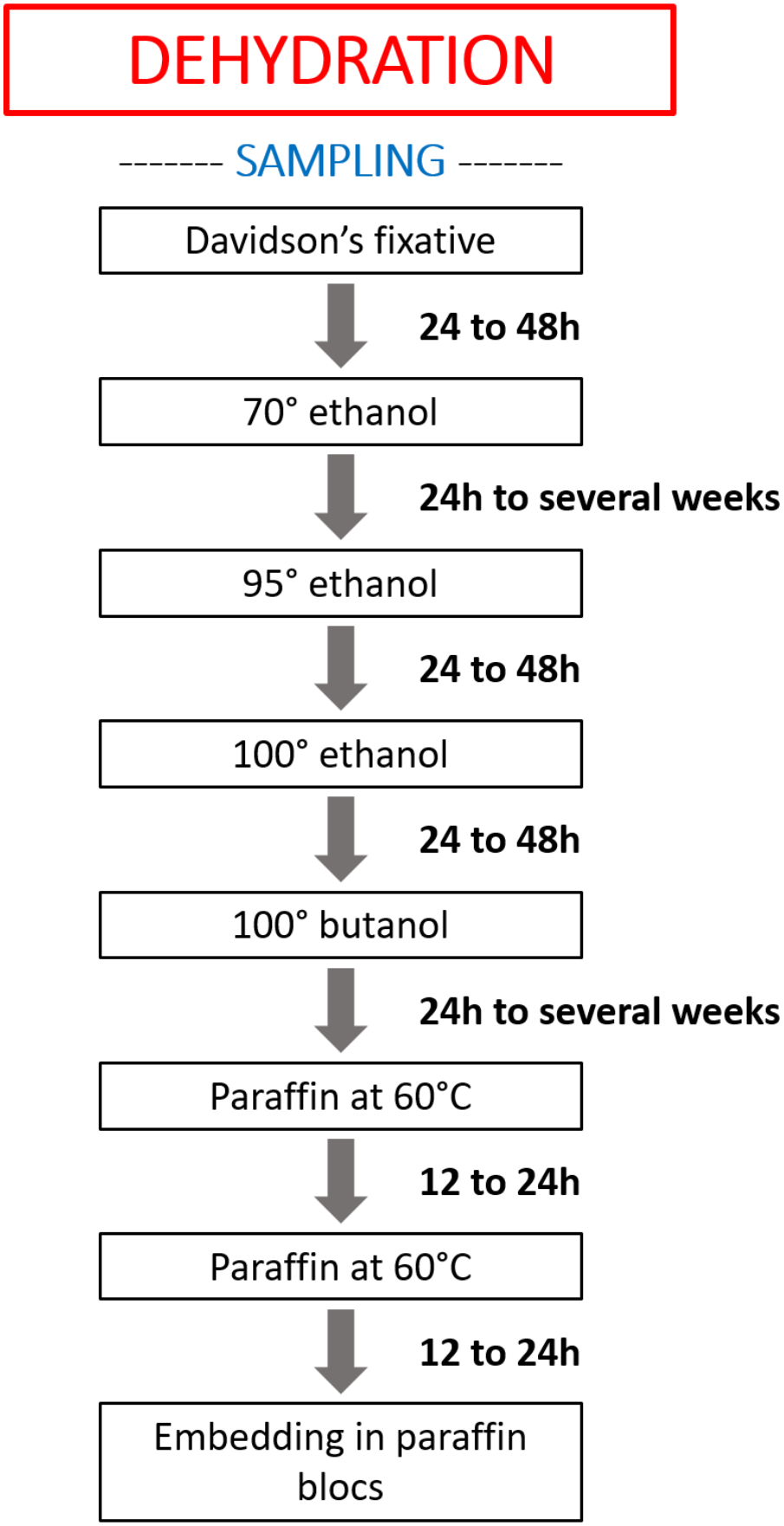


Figure 17. Diagram of the different manual dehydration steps for histological samples of fish gonads

5.2. Embedding in paraffin blocs

Do not forget to place the stainless steel beaker with 500mL of unused paraffin into the 60°C proofer at least 24h before this step.

Handle with nitrile gloves and blouse

Once the second bath of liquid paraffin is finished (*Section 5.1*), samples are ready to be embedded into paraffin blocs.

- Melt paraffin beforehand in a stainless steel beaker in the 60°C proofer.
- **Put the paraffin-filled beaker back into the proofer between each use!**
- Avoid putting your hands on the beaker (generates paraffin cooling and clogging).
- Put thin forceps into the proofer to keep it hot (when heated forceps aren't available).
- **Put the forceps back into the proofer on a paper towel between each use!**
- Take the embedding cassettes out of the proofer one by one to avoid paraffin clogging.

For one sample :

- With long stainless steel forceps, take an embedding cassette out of the paraffin bath from the proofer.
- Place the cassette on a plastic plate, open it, and with the heated forceps, remove the sample and its tag. Replace the forcpes into the proofer.
- Take the stainless steel beaker out and fill one hole of the silicone mould with paraffin up to ¾ (*Fig. 18a*).
- Put the beaker back into the proofer and take the heated forceps.
- With the forceps, place the sample vertically in the center of the mould, the straight edge facing the bottom of the mould (*Fig. 18b*).
- Add the tag on the lip of the mould submerging only the grey section of the tag into the paraffin (*Fig. 18c*).
- Place the forceps back into the proofer and take out the stainless steel beaker to finish the mould with paraffin, until you get a dome of paraffin (*Fig. 18d*).
- Place the beaker with parafin back into the proofer.

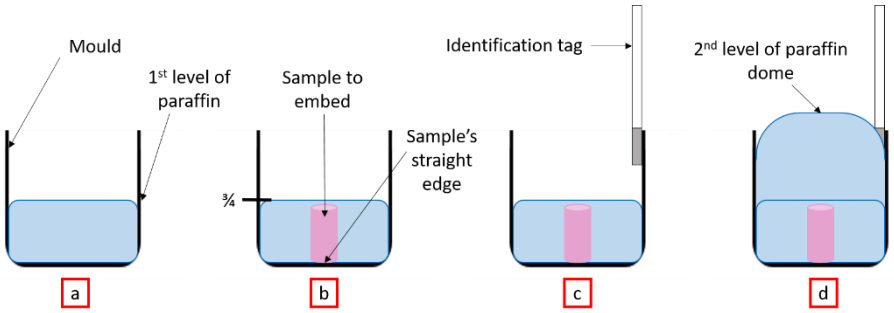


Figure 18. Diagram of the different steps to embed into paraffin: a) Fill the mould to the ¾ of the total volume; b) Place the sample with heated forceps; c) Add the identification tag; d) Finish filling the mould with paraffin until it forms a dome.

If needed, the blocs may be demoulded at least 15 minutes after embedding, once the paraffin has hardened. The mould can be cleared of paraffin and reused right away. The blocs may also be demoulded at a letre time and placed into a refrigerator.

Leave the samples embedded into paraffin blocs in a refrigerator for at least 24h before microtomy!

6. Microtomy

Handling a microtome requires safety training beforehand!

- Keep paraffin blocs on ice before cutting them (may facilitate the cutting process).
- Cut into 3µm sections.
- Set the knife holder to a 5° angle for bigger samples (more than 5mm).
- Use a used blade to remove excess of paraffin for new blocs.
- Use a fresh blade to cut sections to avoid ridges and tears.
- Do not take the first section of the row.
- On a rotary microtome, keep the hand wheel rotation speed constant to avoid folds.
- In the water bath, do not leave the sections more than 2 minutes.
- Choose the nicest sections (complete and unbroken sections of the sample). To separate them, use a strait tip teasing needle on joints between two sections.
- Avoid sections with folds, air bubbles or ridges.
- Avoid placing sections too close to the edge of the slide.
- Place several sections from a single sample per slide.
- Check if the gonadal wall is unbroken! (microscope)

Identify the slides with a pencil and place them into a proofer (37°C) on paper towel covered plate for at least 12h (can stay several days).

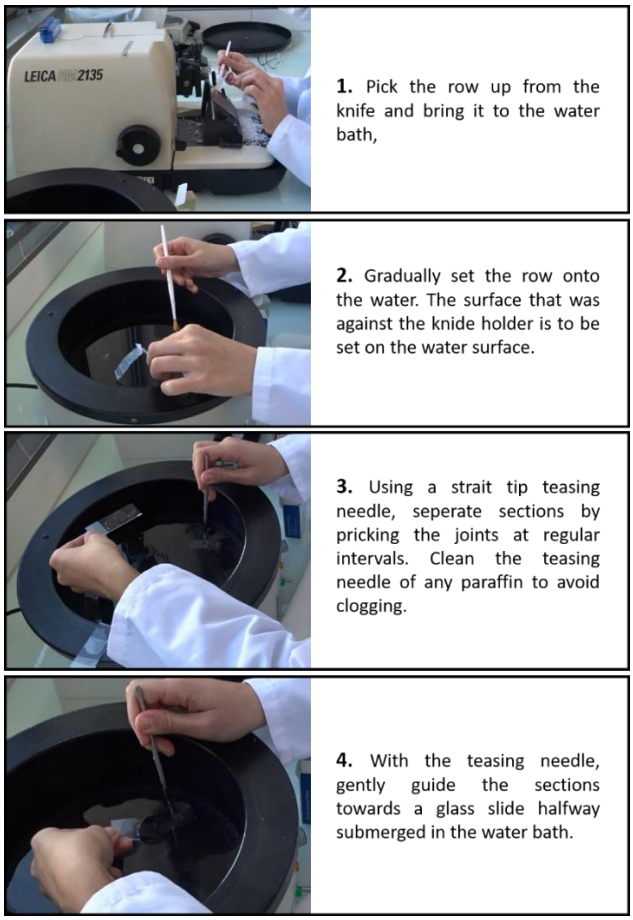


Figure 19. Diagram of the different steps to set the row onto a glass microscope slide.

7. Staining and slide mounting

7.1. Prenant-Gabe trichrome stain

After drying the slides into a proofer at 37°C over night (minimum), they can be stained.

Staining with **Prenant-Gabe's trichrome** highlights connective tissues in green, mucous membranes/cytoplasm in pink and nuclei in grey.

The following steps are carried out under a fume hood with nitrile gloves and a blouse to avoid inhalation of toxic products (alcohols and Diasolv®)

Baths must be prepared in slide staining tanks beforehand (*Fig. 20*). They are filled enough to have the slides completely submerged into the solution when the slide holder (*Fig. 3*) is inserted into the tanks. The tanks are also annotated with the solutions they are filled with and the bathing times (*Fig. 20*). The use of several timers is mandatory. The baths during staining process must be carried out successively.

During distilled water baths, a back and forth movement is made by holding the slides rack into the tank. Flushing with distilled or running water is done with a dropper (*Fig. 21*).

Dewaxing:

1- Diasolv® : 5 minutes
2- Diasolv® : 5 minutes

Hydration :

3- 100° ethanol : 5 minutes
4- 100° ethanol : 5 minutes
5- 95° ethanol : 5 minutes
6- 70° ethanol : 5 minutes
7- Distilled water : Rinse in bath

Staining :

8- 1° Hematoxylin : exactly 1 minute (nuclei staining in grey). Hematoxylin solutions contain hematein and metal mordant (aluminium or iron salts). This mordant is responsible for the staining.
9- Running water: Rinse until the excess color has been removed.
10- Distilled water : Rinse in bath
11- 1° Eosin : 8 minutes (cytoplasm staining in pink). Eosine stains cytoplasm in pink and fibers in a range of different pinks depending on the acidity of different elements.
12- Distilled water : Flushing with a dropper
13- 1° liquid phosphomolybdic acid : 30 seconds (differentiates eosin/fast green)(change after 3 uses)
14- 0.2° liquid fast green: 30 seconds (connective tissues staining in green)

Dehydration :

15- 100° ethanol : 5 minutes (change after every use)
16- 100° ethanol : 5 minutes
17- Diasolv® : 5 minutes (change when necessary)

Figure 20. Plastic tanks noted with the solution they are filled and the bath times.

Figure 21. Flushing with distilled water in a dropper.

Wastes :

- Alcohols + stains, alcohols + Diasolv® are to be disposed in a can A 1993 FLAMMABLE TOXIC LIQUID
- Eosin and fast Green are not toxic. They are to be disposed in suitable containers A 3082 DANGEROUS MATERIAL FOR THE ENVIRONMENT LIQUID
- Hematoxylin is to be disposed in suitable containers A 2810 FLAMMABLE ORGANIC TOXIC

7.2. Slide mounting

Mount slide and coverslip using Diamount® under a fume hood with a blouse and nitrile gloves.

With a disposable pipette, put a few drops of Diamount® on the slides (*Fig. 22*). Progressively, place the coverslip on top, laying it down at an angle to avoid bubbles forming. Do not let the coverslip overtake the sides of the slide.

Place the slide on paper towel in a plate and place it all into a proofer at 55°C for 24h to let the glue polymerise. The slides can later be cleaned and stored away (*Section 8*).

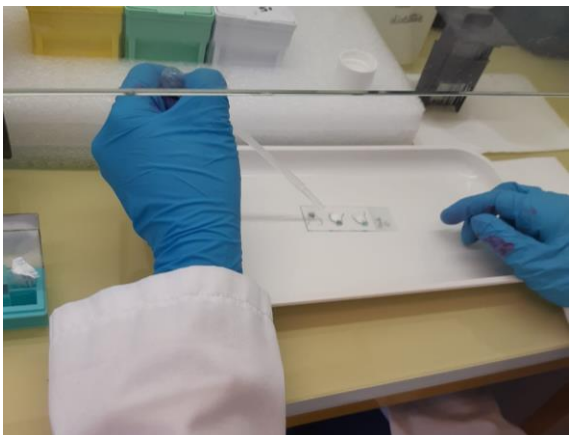


Figure 22. Slide mounting with Diamount®

Cleaning :

- The fume hood can be cleaned with ethanol.
- Broken slides and coverslips must be disposed in a laboratory waste bin.

PRENANT-GABE'S TRICHROME STAIN

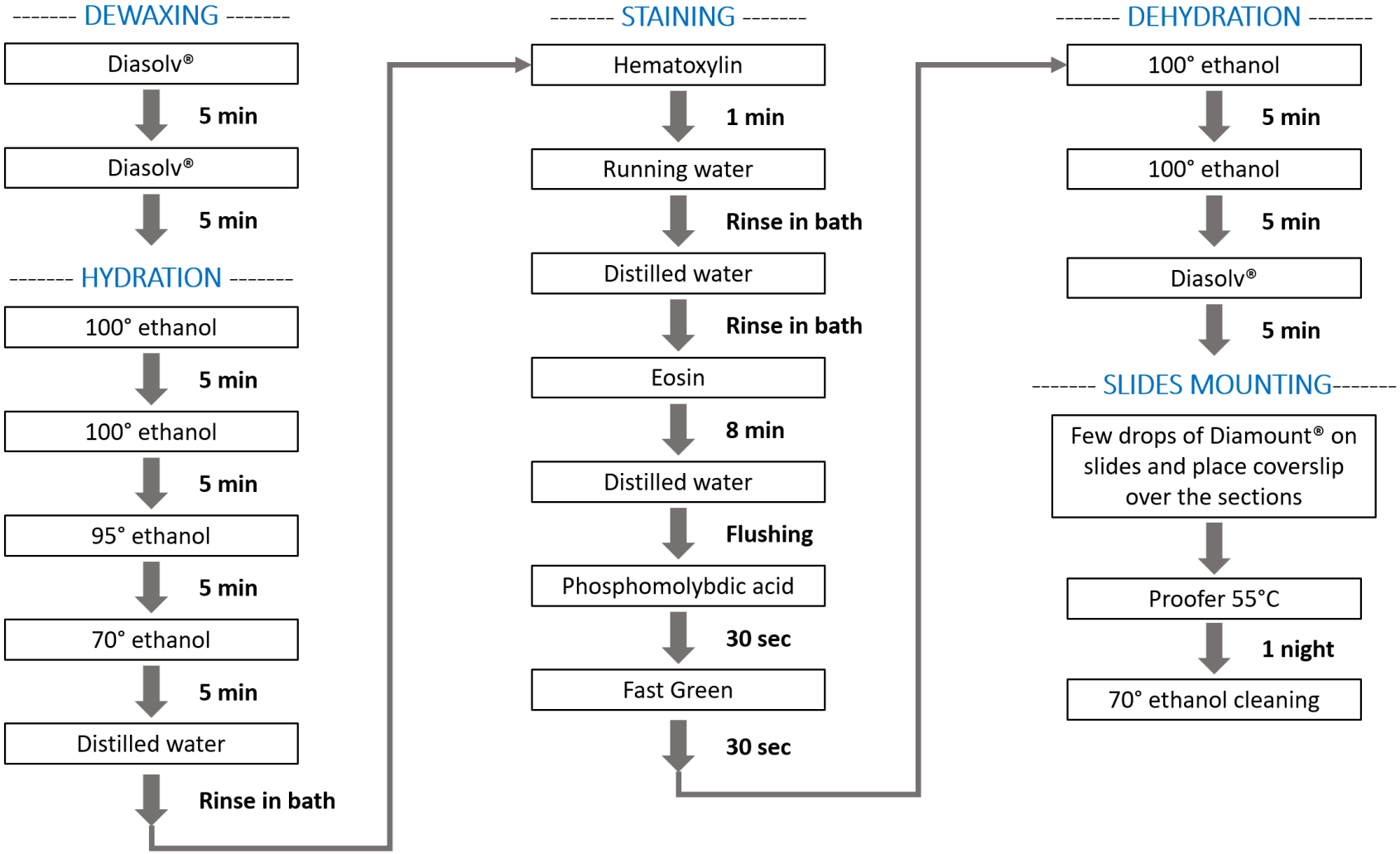


Figure 23. Diagram of the steps used for Prenant-Gabe's trichrome stain.

8. Cleaning, scanning and storage

8.1. Slides cleaning

If there is excess of Diamount® on slides, it is possible to remove the bulk of it by scraping with a razor blade. It is also possible to submerge the slide a few minutes into ethanol to soften the glue and removing it gently with a blade. Finally, clean slides thoroughly with ethanol soaked paper towel before scanning them.

Store slides in histological slides storage boxes to facilitate the transportation and avoid breaking them.

8.2. Slides scanning

It is possible to scan slides in order to keep a virtual copy of the cross sections. The scanner used during the MATO project was the VS120 Olympus model driven by Olympus VS-ASW software, hosted by the CMABIO3. The scans magnification was of x20 and image acquisition was done at three different depths within 3µm (thickness of the paraffin strip). The scanner automatically merged the three images by keeping the clearest pixel between all three depths.

After the overview step on the scanner, the desired scan areas were manually defined. For that, the best cut pattern was selected depending on the following criteria:

- Complete gonadal wall
- No folds, holes, tears
- Staining neither too intense nor too bleak
- Cross sections entirely visible in the scan area

In the event that none of the cuts present on the slide met all of the previous criteria, either prepare a new slide or choose the section with the most material. Once the section is selected, place focus points homogeneously throughout the section while avoiding white areas without biological tissues.

At the end of the scanning process, slides scans are in ".vsi" format. Do not forget to save all output files on an external hard drive at the end of scanning session.

The scanned slides are read with QuPath software (0.3.2 version)(Bankhead et al., 2017). For more detail, see the '**Stereology reading protocol when using quantitative histology for the determination of sexual maturity in fish ovaries'** (Dubroca et al., 2023).

8.3. Paraffin blocs and slides storage

If histological slides are no longer being used, they can be stored in empty cardboard boxes of new glass microscope slide. Annotate each box (species, sex, date, project).

Paraffin blocs can be wrapped with clear tape. Envelope the tape 1½ times around the bloc, covering the area that was cut with the microtome (with the sample in sight) and the tag. Be careful to tape the tag in the right position in order to be able to read the sample's ID!

Moreover, it is advised to store blocs in « ziploc » bags, correctly annotated (species, sex, date, project). The bags can then be stored in a cardboard box, in a dry, cool and ventilated room.

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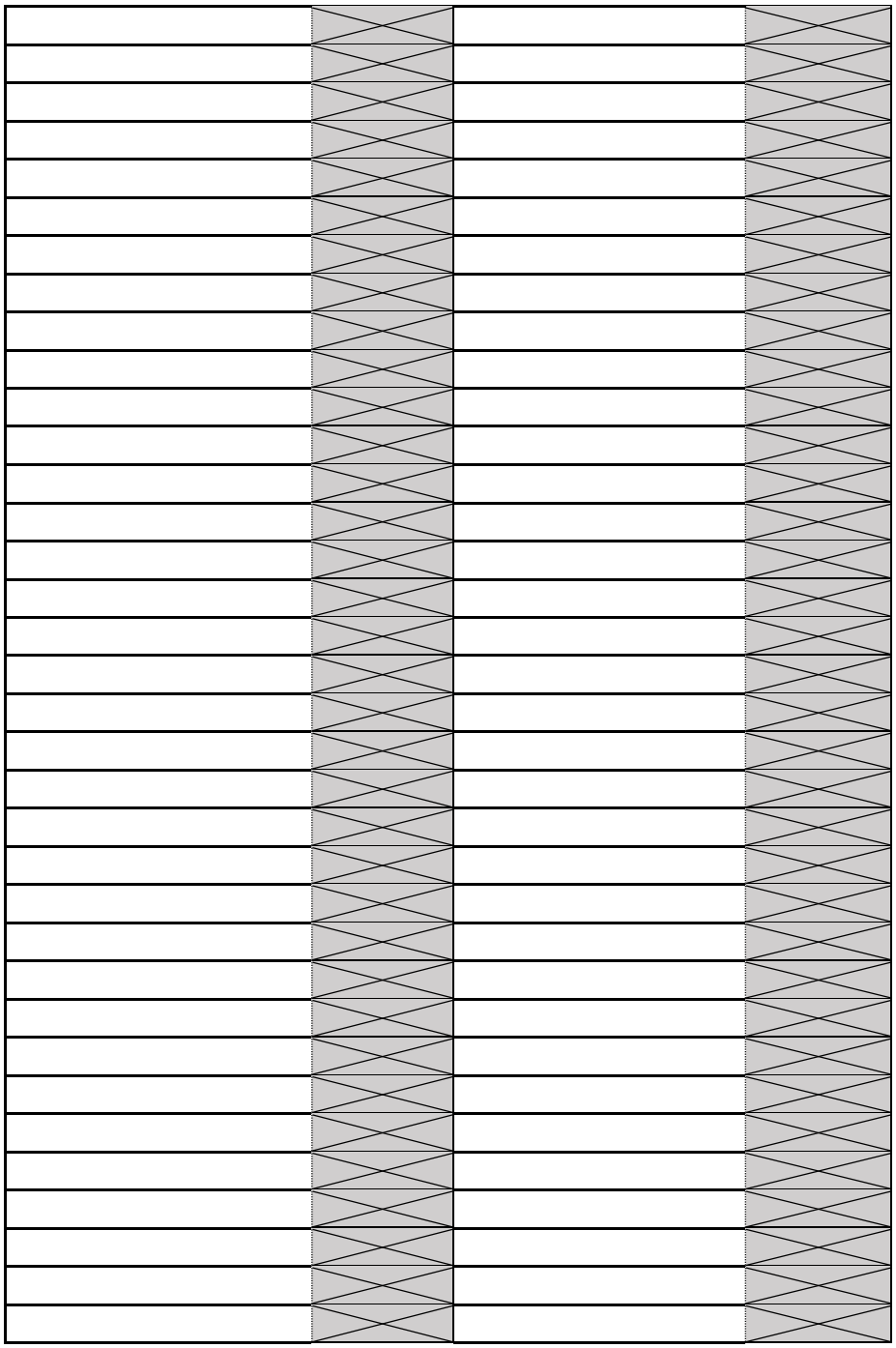
Annexes

Annex 1 : Identification tags for tissue processing embedding cassettes



Annex 2 : Data entry sheet

ID Fish	Species name	ICES division	ICES stat rectangle	Date	Total length of the fish (cm)	Ungutted fish weight (g)	Parasites pressence	Age (year)	Visual maturity	Liver weight (g)	Dorsal/Right gonad weight (g)	Ventral/Left gonad weight (g)	Number of sections	Vessel

Annex 3 : Gay-Lussac's table

Table for dilution of alcohol (Gay-Lussac's table)

		Initial concentration													
		100	99	98	97	96	95	90	85	80	75	70	65	60	50
Final concentration	95	6,5	5,15	3,83	2,53	1,25									
	90	13,25	11,83	10,43	9,07	7,73	6,41								
	85	20,54	19,05	17,58	16,15	14,73	13,33	6,56							
	80	28,59	27,01	25,47	23,95	22,54	20,95	13,79	6,83						
	75	37,58	35,9	34,28	32,67	31,08	29,52	21,89	14,48	7,2					
	70	47,75	45,98	44,25	42,54	40,85	39,18	31,05	23,14	15,35	7,64				
	65	59,37	57,49	55,63	53,81	52	50,22	41,53	33,03	24,66	16,37	8,15			
	60	72,82	70,8	68,8	65,85	64,92	63	53,65	44,48	35,44	26,47	17,58	8,76		
	55	88,6	86,42	84,28	82,16	80,06	77,99	67,87	57,9	48,07	38,32	28,63	19,02	9,47	
	50	107,44	105,08	102,75	100,44	98,15	95,89	84,71	73,9	63,04	52,43	41,73	31,25	20,47	
	45	130,26	127,67	125,11	122,57	120,06	117,57	105,34	93,9	81,38	69,54	57,78	46,09	34,46	11,41
	40	158,56	155,68	152,84	150,02	147,22	144,46	130,8	117,34	104,01	90,76	77,58	64,48	51,43	25,55
	35	194,63	191,39	188,19	185,01	181,85	178,71	163,28	148,01	132,88	117,82	102,84	87,93	73,08	43,59
	30	242,38	238,67	234,99	231,33	227,7	224,08	206,22	188,57	171,05	153,61	136,04	118,94	101,71	67,45
	25	308,9	304,52	300,18	295,86	291,56	287,28	266,12	245,15	224,3	203,61	182,83	162,21	141,65	100,73
	20	408,5	403,13	397,79	392,47	387,17	381,9	355,8	329,84	304,01	278,26	252,58	226,98	201,43	150,55
	15	574,75	567,43	560,53	553,55	546,59	539,66	505,27	471	436,85	402,81	368,83	334,91	301,07	233,64
	10	907,09	896,73	886,4	876,1	865,15	855,15	804,5	753,65	702,89	652,21	601,6	551,06	500,5	399,85

Black numbers indicate the amount of water in mL to be added to 100mL of alcohol of initial concentration of x (in blue) to obtain the desired concentration.

Example: The table indicates that 105,34mL of water should be added to 90° alcohol to obtain 45° alcohol.
Warning: The final volume is less than the sum of the involved volumes. This is the phenomenon known as « volume concentration », variable depending on the title of the initial alcohol.

- Handle ethanol under a fume hood with nitrile gloves and protective clothing

Annex 4 : Davidson's fixative

For 4L of Davidson's fixative, add in the following order :

- 400ml of glycerol
- 800ml of formaldehyde
- 1200ml of 95° ethanol
- 1200ml of filtered sea water
- 360 ml of acetic acid

- Add the acetic acid at the last moment before use.
- Store pre-Davidson's fixative (without acetic acid) several months in a refrigerator.
- Store Davidson's fixative several weeks.

- Handle under a fume hood with nitrile gloves and protective clothing.
- Samples must be completely submerged
- Do not exceed 1.8L per sample bottle (to avoid accidental spills during manipulations)
- Sample bottles must be correctly identified (date, project's name, species, chemical product)
- Store at 4°C, with or without samples
- When transferring samples from Davidson's fixative to 70° ethanol, if Davidson's fixative is turbid (used for more than 120 samples and/or for ovaries rich in hydrated oocytes), empty it into the appropriate chemical waste bin (chemical waste bin for flammable liquids with safety funnel) to facilitate picking up the tissue processing embedding cassettes.

- Since Davidson's fixative is a dangerous substance (classification H315, H319, H317, H341 and H350 according to the EC Regulation n°1272/2008), it must be handled under a fume hood with Nitrile gloves and appropriate protections (blouse, safety goggles, closed shoes,…)
- Avoid inhalation of toxic products (Davidson's fixative) 
- Sample bottles must be stored in an appropriate storage : DO NOT STORE THEM IN A REFRIGERATOR WITH PRODUCTS FOR CONSUMPTION

Annex 5 : Prenant-Gabe's trichrome stain

- **0.2° Fast Green :**
  - Fast Green (powder) **2g**
  - Acetic acid ($C_2H_4O_2$) **10 drops**
  - Distilled water **QSP 1L**

Green color.

- Handle under a fume hood with nitrile gloves, protective clothing and closed shoes.
- Filter before use.
- Store indefinitely in the dark.
- Use a brown glass bottle or aluminium-covered vials to store products in the dark.
- Reusable after each staining.

- **Hematoxylin :**
  - Solution A :
    - Distilled water **500ml**
    - Concentrated sulphuric acid (H_2SO_4) **8ml**
    - Ammonium Iron III sulfate ($H_4NO_8S_2Fe$, $12H_2O$ – MW=482.19) **10g**
  - Solution B :
    - 95° ethanol **500ml**
    - Hematoxylin **5g**

Mix A + B. Wait 30 minutes (residue).
Brick red color.

- Handle under a fume hood with nitrile gloves, protective clothing and closed shoes.
- Filter before use.
- Store 2-3 months in the dark.
- Use a brown glass bottle or aluminium-covered vials to store products in the dark
- Change when dark brown.

- **1° Eosin :**
  - Eosine Y (or B) **10g**
  - Distilled water **QSP 1L**

Red color.

- Handle under a fume hood with nitrile gloves, protective clothing and closed shoes.
- Filter before use.
- Store indefinitely in the dark.
- Use a brown glass bottle or aluminium-covered vials to store products in the dark.
- Reusable after each staining.

- **1° Phosphomolibdic acid :**
  - Phosphomolibdic acid ($H_3[P(MO_3O_{10})4]$, $10H_2O$ – MW = 1824.96) **10g**
  - Distilled water **QSP 1L**

Yellow color.

- Handle under a fume hood with nitrile gloves, protective clothing and closed shoes.
- Store indefinitely in the dark.
- Use a brown glass bottle or aluminium-covered vials to store products in the dark.
- Change after few staining.