PROTOCOLS FOR SEA ANEMONE HISTOLOGY

Estefanía Rodríguez

1) PARAFFIN EMBEDDING PROCESS (modified from Pantin 1948)

Because they have soft bodies, paraffin tissue embedding is necessary in order to histologically section selected parts of sea anemones. Selected pieces of specimens are submerged in a series of reagents. Standard procedures for the study of sea anemones include histological preparations of longitudinal sections of the distal and proximal column, and cross section of the column at the actinopharynx level and tentacles; in different species additional specific parts of the specimens might need histological preparations. Step A (dehydration step) consists on a series of increasing ethanol concentrations to slowly dehydrate the tissue. Step B (Paraffin infiltration**)** prepares the tissue for and allows the paraffin to slowly infiltrate the tissue. Once the infiltration is finish, proceed to make paraffin blocks with selected pieces inside, allow them to cool and solidify prior to sectioning them with a paraffin microtome.

A) Dehydration steps	**B) infiltration steps**
Ethanol 70%	50:50 Ethanol 100%: Safeclear*
Ethanol 70% (80%)	Safeclear I
Ethanol 80%	Safeclear II
Ethanol 90% (96%)	50:50 Safeclear: Paraffin
Ethanol 100%	Paraffin I
Ethanol 100%	Paraffin II

Transfer the tissue from one solution to the next, proceeding down the list. Different times are necessary for different sizes of animals: thin (15 min/step[1], total 3h); small (35 min/step, total 7h); medium (25 min/step, total 5h); large (1h/step, total 12h).

*xylene substitute (less toxic)

[1]All steps except for the paraffin steps (use more time there)

2) MOUTING PRIOR TO STAINING

After obtaining 5-10 μm thick sections (with a microtome), these have to be mounted on slides prior to the staining process. Sections can be mounted in a hot plate or hot water bath (temperature should be 5-10°C below the melting point of paraffin to avoid paraffin melting). If using a hot plate, spread a thin layer of solution of albumin (mix of egg white and glycerin (1:1)) or of gelatin powder in water on the slide before the section is positioned on it; let the solution air dry before staining. If using a hot bath, gelatin powder or agar could be use in the water bath; in this case, drop the section in the water bath, let it stretch for several minutes; once the section is stretched, submerge the slide under it and slowly take it out of the bath; the section will stick to the slide. Let the section air dry.

3) STAINING

Histological sections need to be stained in order to facilitate the interpretation of the different structures. There are many different staining protocols. For sea anemones trichromic stains are preferred but not necessary. Here I provided three different options; colors of tissues and structures will vary depending on the chosen protocol. Because chosen stains are soluble in water, sections need to go through a hydration step (A) in order for the stains to penetrate into the tissue. For storage, sections are covered and sealed with natural or synthetic resins (see section 4 below) of hydrophobic nature; this requires a rapid dehydration process (B) in each staining protocol provided.

3.1) HEIDENHAIN AZAN STAIN (Presnell & Schreiban 1997)

The results of this stain are as follow:

nuclei…….dark red
erythrocytes……...orange
collagen fibres, cartilage matrix and mucus…….blue

A) Hydration steps

Safeclear I	(10 min)
Safeclear II	(10 min)
Ethanol 100%	(3 min)
Ethanol 100%	(3 min)
Ethanol 95%	(3 min)
Ethanol 70%	(3 min)

B) Stain and dehydration steps

Acid alcohol	(3 min)
Azocarmine*	(30 min) T: 50°C
DH_2O (dip)	
Aniline alcohol	(3 min)
Phospho acid*	(30 min)
DH_2O (dip)	
Aniline blue	(1.5 min)
Acid H_2O	(1.5 min)
Ethanol 80% (dip)	
Ethanol 95%	(1.5 min)
Ethanol 100%	(3 min)
Ethanol 100%	(3 min)
Xylene	(5 min)

***Process can be stopped in these steps for a while

Solutions:

Azocarmine

Azocarmine G ……..0.2 to 1.0 g
Distilled water ……..100.0 ml
Glacial acetic acid ……..1.0 ml

Boil azocarmine in water 5 min, cool, filter, and add acetic acid

Aniline alcohol

Aniline ……....1.0 ml
85-90% alcohol …….1000.0 ml

Acid alcohol

Glacial acetic acid ……..1.0 ml
90-95% alcohol ……..100.0 ml

Phosphotungstic acid

Phosphotungstic acid ……..……..5.0 g
Distilled water ……..100.0 g

Aniline blue stain

Aniline blue WS ……0.5 g
Orange G……2.0 g
Oxalic acid……..2.0 g
Distilled water ……..100.0 ml
5% phosphotungstic acid ……..1.0 ml

Acidified water

Distilled water ……..100.0 ml
Glacial acetic acid ……....1.0 ml

3.2) RAMON Y CAJAL TRICHROME (Gabe 1968)

The results of this stain are as follow:

epidermis and gastrodermis……..pink,
mesoglea ……..blue
muscle fibers…….green.

A) Hydration step

Safeclear I	(10 min)
Safeclear II	(10 min)
Safeclear III	(10 min)
Ethanol 100%	(5 min)
Ethanol 96%	(5 min)
Ethanol 70%	(5 min)
DH_2O (dip)	(2 min)

B) Stain and dehydration step

Acid fuchsine	(5-10 min)
Acidified water (dip)	
Picricindogocarmine acid	(10 min)
Ethanol 70%	(rinse once/twice)
Ethanol 95%	(rinse once/twice)
Ethanol 100%	(rinse once/twice)
Ethanol 100%	(rinse once/twice)
Xylene	(5 min)

Solutions:

Acidified water

Distilled water ……..100.0 ml

Glacial acetic acid ……....1.0 ml

Basic fuchsine (48h life)

Basic fuchsine stock solution……..10 ml
Distilled water ……...100.0 ml

Discard solution after 48 hours.

Ziehl fuchsine phenic stock solution (for 104 ml)

Basic fuchsine ……..1.0 g
Distilled water ……..90.0 ml
96% ethanol ……..10.0 ml
Phenol……5 g/4.65 ml (liquid phenol 90%, PM/FW=94)

Picricindogocarmine acid

Picric acid saturated solution (*)……....100 ml
Indigocarmine ……..….0.4. g

(*) Saturation point 13gr/L (for 100 ml of solution, add 1.3 gr picric acid powder). Add water and filter.

3.3) MAYER HEMATOXYLINE & EOSIN (Mallory 1944)

The results of this stain are as follows:

collagen.....pale pink
muscle.....deep pink
acidophilic cytoplasm.....red
basophilic cytoplasm.....purple
nuclei.....blue
erythrocytes.....cherry red

A) Hydration step

Safeclear I *	(10 min)
Safeclear II	(10 min)
Safeclear III	(10 min)
Ethanol 100%	(5 min)
Ethanol 96%	(2 min)
Ethanol 80%	(4 min)
Ethanol 70%	(2 min)
Distilled H_2O	(2 min)

B) Stain and dehydration step

Hematoxyline sol.	(20-40 min)
H_2O running	(10 min)
Acidic alcohol 5%	(dip)
H_2O running	(10-15 min)
Eosin solution	(3 min)
Distilled H_2O	(dip)
Ethanol 70%	(~2 min)
Ethanol 90%	(4 min)
Ethanol 96%	(2 min)
Ethanol 100%	(2 min)
Ethanol 100%	(2 min)
Xylene	(5 min)

***additional way is to have the slides in the stove for 10 min and then xylene or substitute for 5 min**

Solutions:

Acid alcohol 5%

Ethanol 70% ……..100.0 ml
HCl (Hydrochloric acid)…….5.0 ml

Hematoxylin Solution (Mayer):

Hematoxylin ……1 g
Sodium iodate ……. ..0.2 g
Potassium alum……..50 g
Citric acid ……1 g
Chloral hydrate……..50 g

Add 1 g of hematoxylin to 1 L of distilled water. Heat gently and add sodium iodate and potassium alum. Heat until dissolved; add citric acid and Chloral hydrate. Allow to ripen, probably for 6-8 weeks, although it can be used within 1-2 weeks.

Eosin Solution:

Eosin Y…… 1.0 g
70% alcohol…….1000 ml
Glacial acetic acid……5.0 ml

Dilute with equal volume of 70% ethanol for use and add 2-3 drops of acetic acid

4) MOUTING PROCESS AFTER STAINING FOR SECTIONS

Stained sections have to be sealed for observation and storage. Natural (e.g. Canada Balsam) or synthetic resins (e.g. DPX, eukitt, etc.) can be used. Synthetic resins are preferred because they dry quicker and do not turn yellow or crack after years.
Cover the slide with the stained section with a slide cover after adding Canada Balsam (diluted slightly with xylene, to thin the balsam) on the surface of the slide cover. Push air bubbles out (pressing carefully) and let dry.

5) PERMANENT SLIDES FOR CNIDAE MEASUREMENT

Put 1-2 drops of lactophenol (*) solution on a slide. Get a little bit of tissue of the desired part of the anemone (scratching the body part) on the slide and cover it with a cover slip. Smear the tissue in the slice so it gets distributed and not concentrated in one spot. Dry the excess lactophenol along the edges with a paper tissue and seal the preparation (painting around the edges) with synthetic resin (e.g. Entellan, DPX, or nail polish as a substitute). Label the slice and let it dry.

Lactophenol (*)

Lactic acid……. 1 part (clears the tissue)
Phenol…… 3 parts (avoids microorganisms growth)
Glycerin……. 2 parts (thickens the mix)
Distilled water…… 1part

(*) Because of the phenol this is toxic, handle with caution.

6) PERMANENT SLIDES FOR CNIDAE MEASUREMENT OF DISCHARGED CNIDAE (Yanagi's method 1999)

Scratch a small amount of tissue from the specimen with the tip of tweezers or a knife and put it into a drop of 4% acetic acid or HCl solution on a slide. After one to two minutes drawn off the liquid carefully with a tissue. Add a solution 1:1 of seawater: glycerin with a few drops each of phenol and formalin per 100 ml. Sealed the slide.