BC Children's Hospital™ Research Institute

Best practices-Histology

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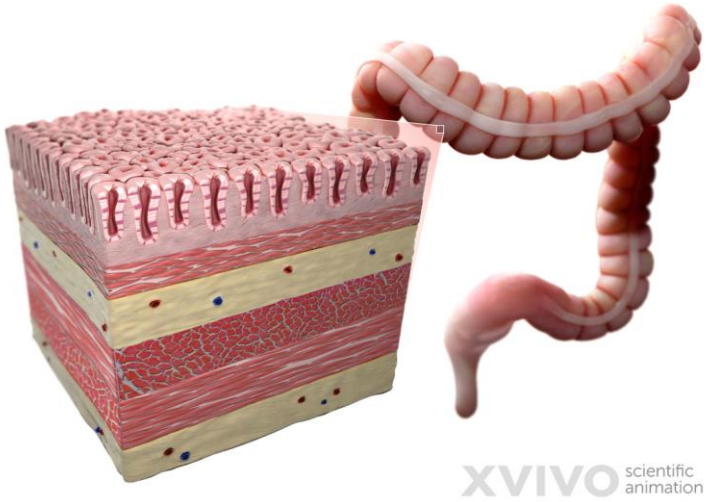
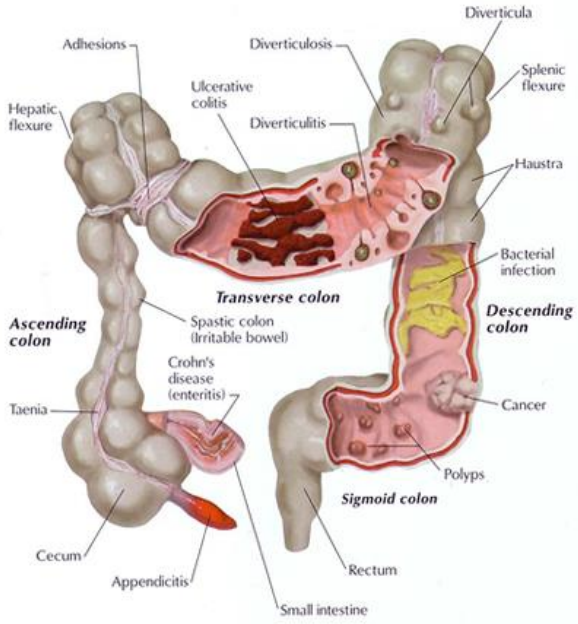
Why we should care about histology?

Histology means the science of the tissues (role, anatomy, interaction)
histos *is Greek for tissue (or web)* ***logia*** *is Greek for branch of learning*

Tissue was first used to describe the different textures of **body parts.** They comprise the **Building Blocks** of our bodies.
Tissue are made of cells clustered to complete a shared function. From tissues arise organs, and organs keep the body operating.



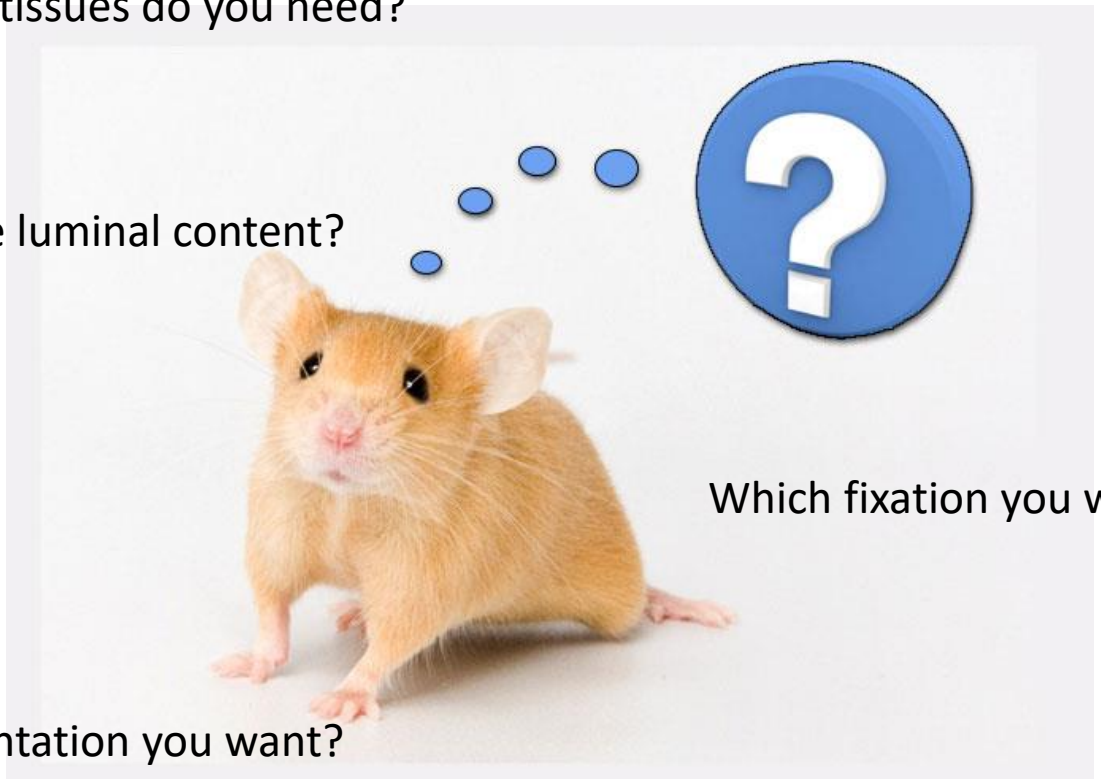


Histology can : - Understand how cellular behavior influences tissue function.
- Predict and understand organ behavior and function.
- Observe how tissue is affected by disease.

First thing to do : Strategic Plan

Which tissues do you need?

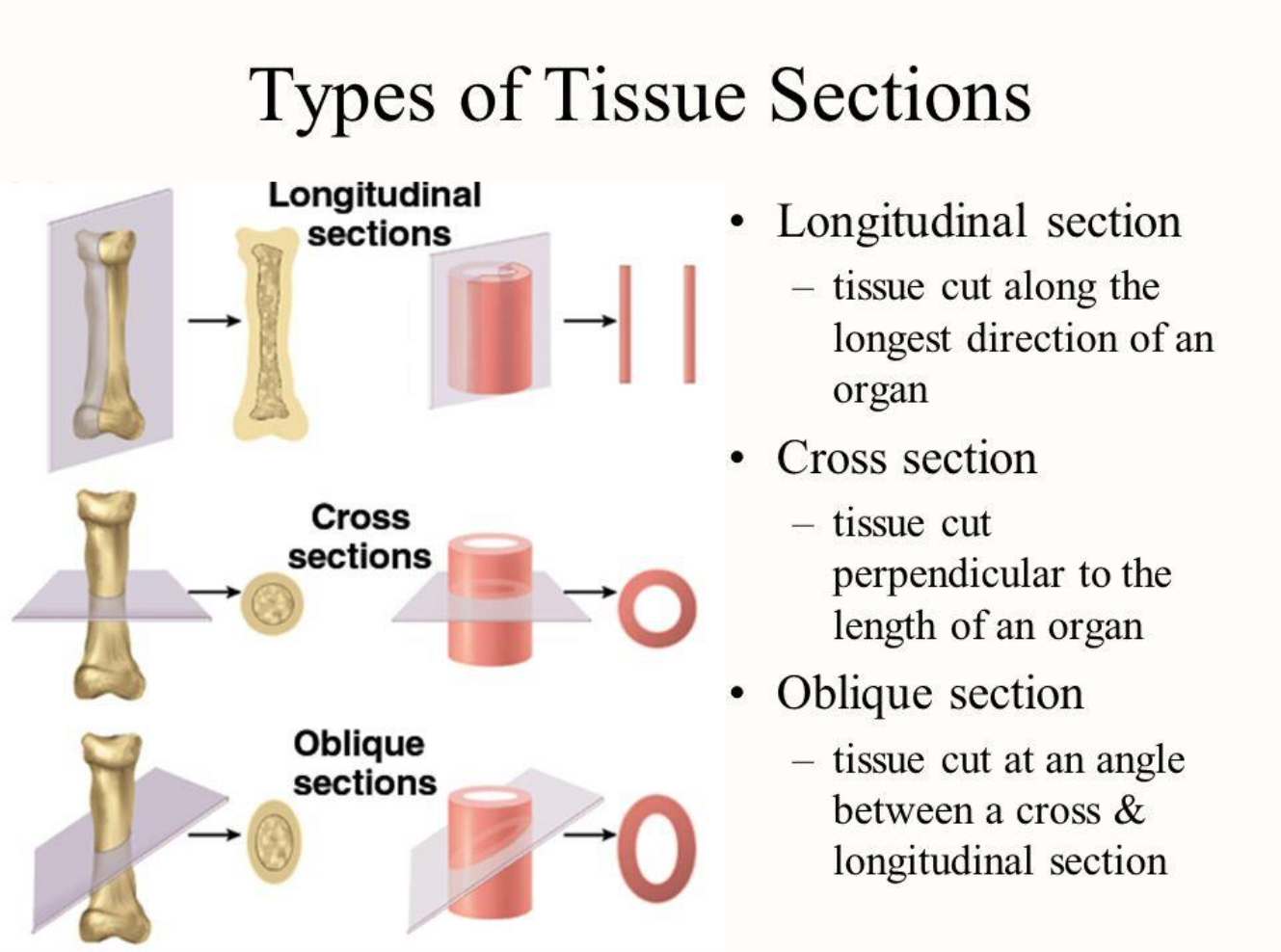
Is it useful to preserve luminal content?



Which fixation you will use?

Which orientation you want?

Tissue Orientation



Longitudinal sections:
Swiss roll
Tube
Colon sheet

Cross (transverse) sections :
Donut or
Bundling

For gut studies, **avoid oblique section** since they will give you bad oriented tissue and uneven layers (muscular or epithelium).

Good Tissue preparation = Good Tissue Orientation = Excellent Slides

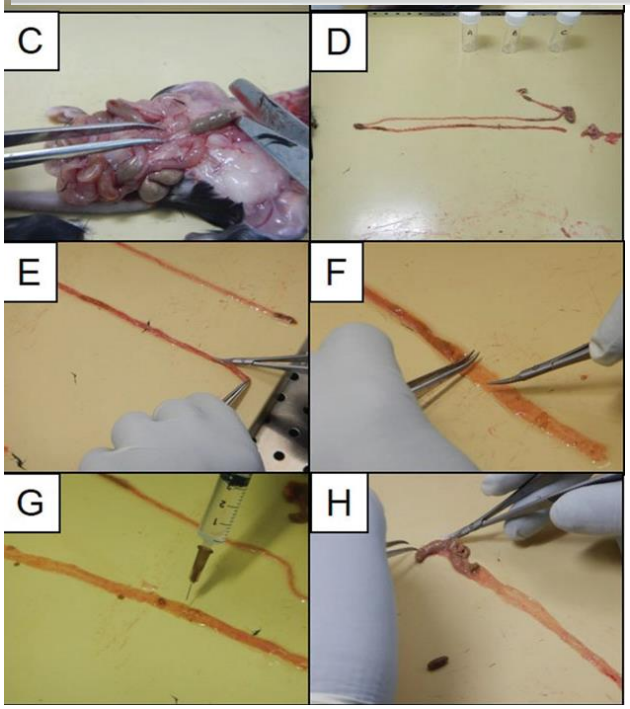
Tissue preparation for good orientation

Good surgical tools are essential to conserve tissue architecture *(sharp, clean, smooth = not twisted or broken)*

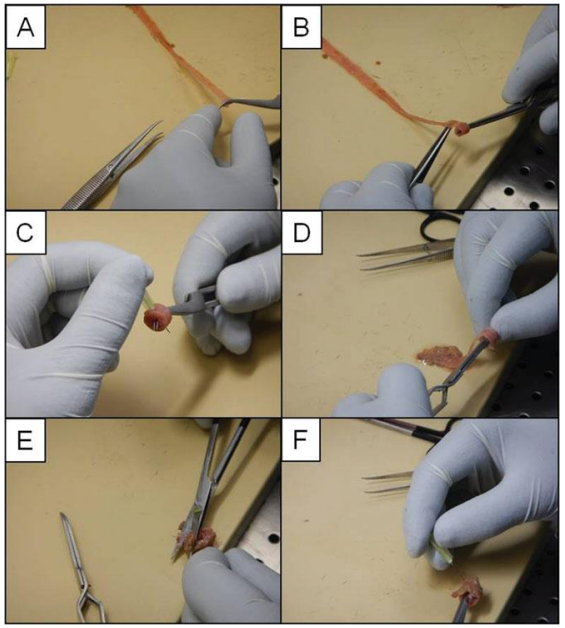
To open the tube :
Use sharp scissors
Keep a steady, gentle tension with the tweezer
Keep the cutting as smooth as **possible.**
Helps to give a straight edge;

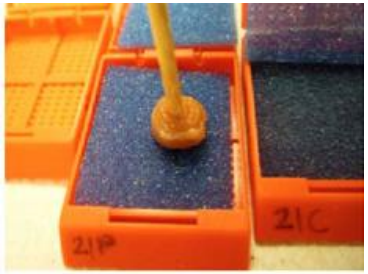
an uneven edge = difficult to keep intestine flat and roll well.

Do not touch the mucosal surface with the **curved tip of the forceps/tweezers**, but **guide them along the lumen to roll back the edge of the intestine** (have a flat tissue) **and clear away any feces**.



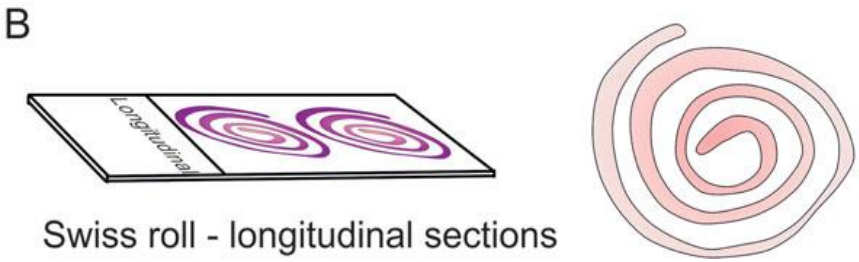
Longitudinal section – Swiss roll





Pros :
longitudinal continuity of tissue

Cons :
Hard to maintain Swiss roll during fixation.
Need straight edge to have high quality sections

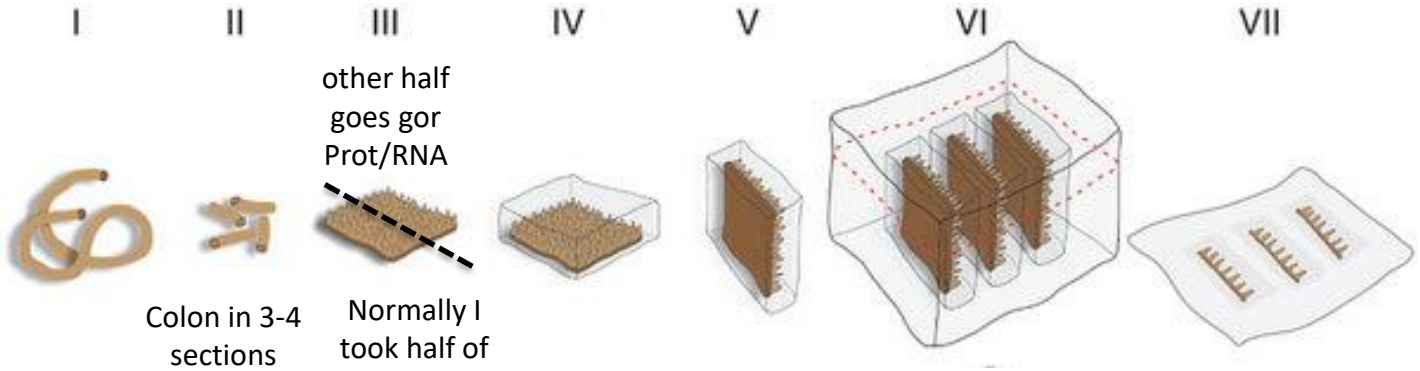


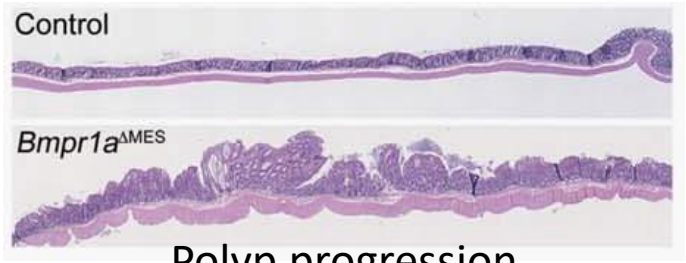
Procedure
Roll the intestine around a toothpick.
Quickly put in fixative (in a tube) = Keep the roll intact compare to a cassette (compress tissue)
Ideal for cancer progression studies or DSS treatment (patchiness of treatment)

http://mousepheno.ucsd.edu/overview.shtml

Moolenbeek and Ruitenberg, 1981

Longitudinal section – Colon Sheet





Polyp progression

DSS treatment

Pros :

Better fixation compared to whole gut tube longitudinal continuity of tissue

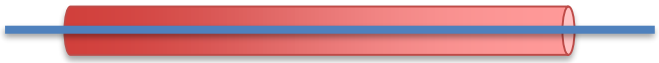
Better tissue orientation = good preparation

Cons :

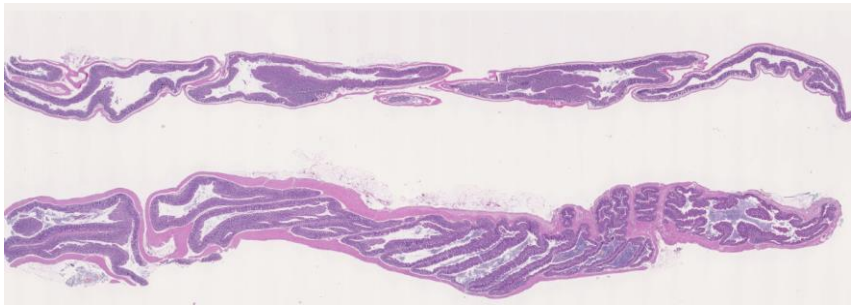
need to be flattened between histology **sponges (biopsy foam pads)** into cassettes, before fixing to give better slides.

Instruction needed to histology to embed the sheet upright

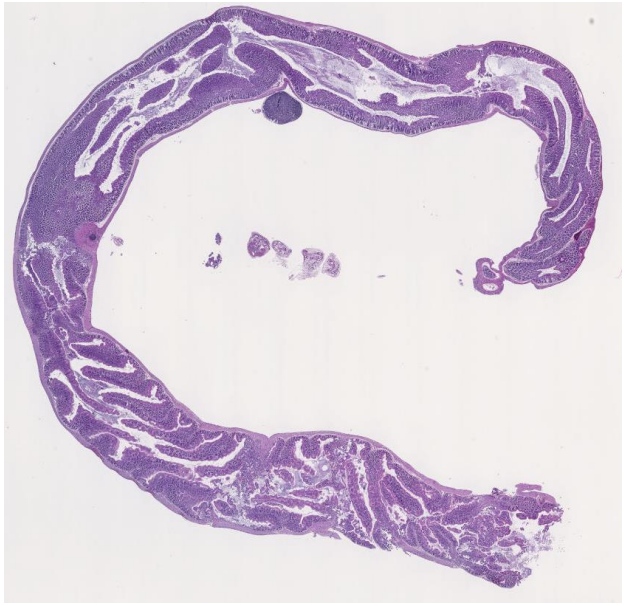
Longitudinal section – Tube

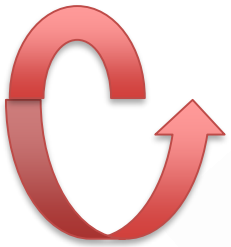


Tube as a sheet



Tube as a Swiss roll





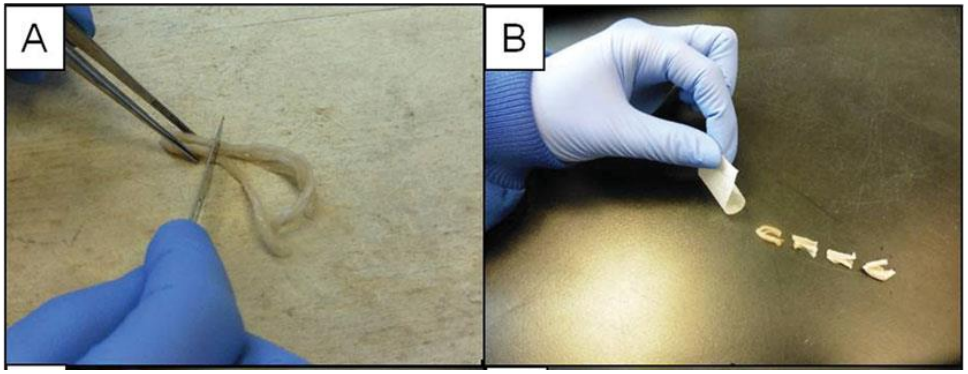
Tube as a Swiss roll

Pros :
Complete longitudinal continuity
Easy process for quick assessment of histology

Cons :
More difficult to optimize tissue architecture / orientation and longitudinal continuity
Interior fixation problem
Needs to be flattened between histology sponges **(foam pads)**

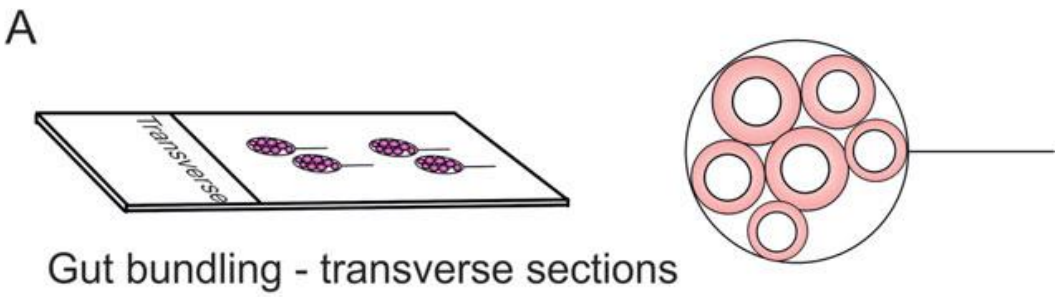
Cross Sections - Donut

The gut bundling technique (donut) was first developed for the study of radiation-induced crypt apoptosis and proliferation. This technique optimized histology and quantitative analysis of cell behavior and cell scoring.



Pros :
Rapid immersion in formalin
Less tissue handling,
Less compression
Less distortion of villi,
Better preservation of apoptotic cells at villi tip

Cons :
Snapshot of a specific region
Can miss a event occurring 50um higher
Not adequate for cancer or DSS study

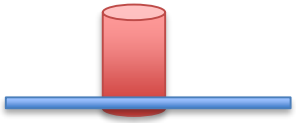


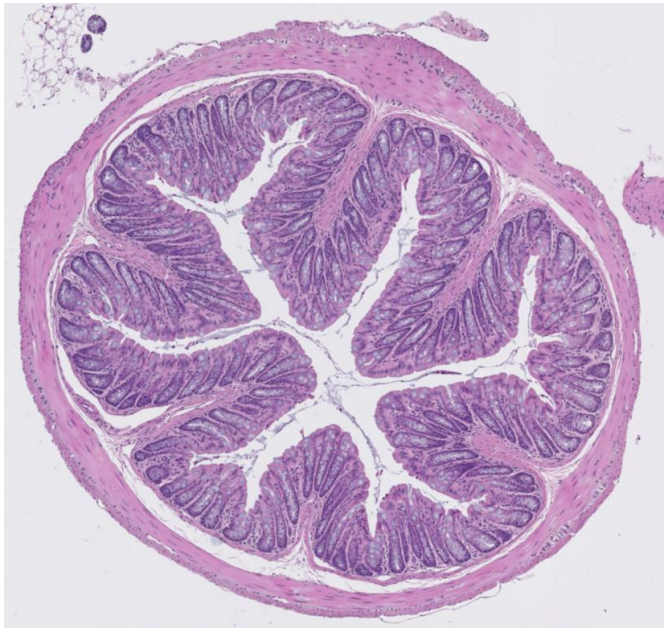
Duckworth and Pritchard, 2009; Duckworth et al., 2013; Williams et al., 2013; Burkitt et al., 2015)

(Potten et al., 1990; Booth and Potten, 2002).

Cross Sections - Donut

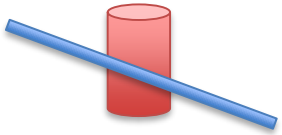
***** Always use sharp blades or scissor to cut donut. Make sure to create Blunt end (straight edge)**

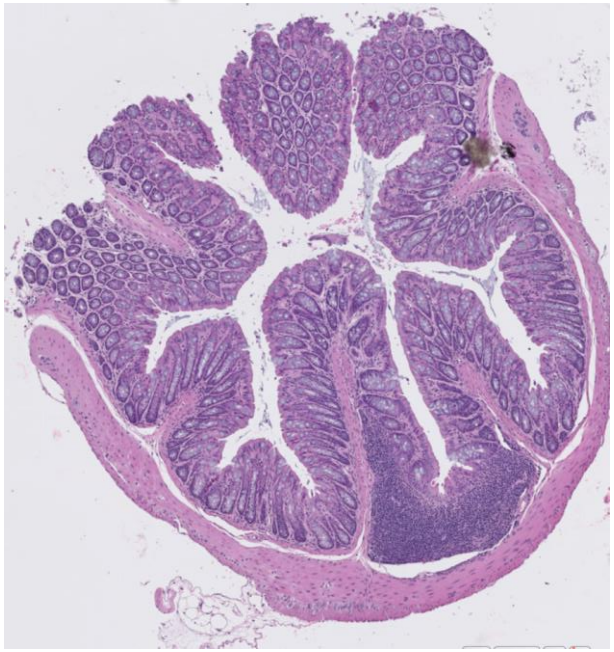




Perfect cross-section
Blunt end cut.
Both Muscularis layer in a circular manner

or





Can be cause by not straight edge
no Blunt end = oblique sections

Aldehyde fixatives

Fix tissue (i.e., arrest biological degradation) by forming chemical “cross-links” between and within proteins.

Formalin 10% Neutral Buffered Formalin (NBF) : Most commonly used since read-to-use and convenient. Stable in neutral buffer.

4% Paraformaldehyde (PFA) : Superior fix for IHC as it tends to reduced background staining.
Must be freshly made just before used. Less convenient than NBF.

Gluteraldehyde : Mostly used for Electron Microscopy

Procedures :
Dissect the tissue as rapidly as possible and immerse in at least 20X volume of fix to tissue.
Fix ‘**overnight**’ (14 to 20 hours) at **4°C.**
Wash the tissue in several changes of 70% EtOH and placed in 70% ethanol for extended storage. (prevents Bacterial degradation)

Blind, C, et al J Clin Pathol 2008;61:79–83;

Fergenbaum, JH, et al Cancer Epidemiol Biomarkers Prev 2004;13(4).

Problem with Aldehyde fixation

Risk for **under-fixation** and for **over-fixation**

Penetration rate of Aldhehyde fixative : **2-3mm / hour**
It's variable depending on the density and other characteristics of the tissues.

Fixing large pieces of tissue increase risk of under-fixing the interior (Eg. Liver or intestinal tube (not open)).
For that kind of tissue, the size should not exceed 10-15mm (1-1,5cm).

For very **small pieces of tissue** (~1-2mm range dimensions) an overnight fix may be too long. **One to several hours** may be a better choice for this material but be aware that changing times of fixation between experiments may have serious effects on subsequent immunostains.

Fixation method / time / temperature is a variable that needs to be considered when developing immunohistochemistry protocols.

**** Please try to be consistent (in fixation conditions) to reduce variable staining results.**

Example of Under Fixation

** Minimize and **standardize** the time taken between death and tissue fixation

** Be **consistent** in fixation incubation

The intestinal mucosa is **extremely sensitive** (after death) :

- to prolonged periods of **drying**
- to undergoes rapid **autolysis** (self-digestion)
- to **sloughing** of the epithelium
- to **artifactual** changes

Tissue must be **fixed as quick** as possible and **keep on ice** during sample harvesting.

http://mousepheno.ucsd.edu/overview.shtml

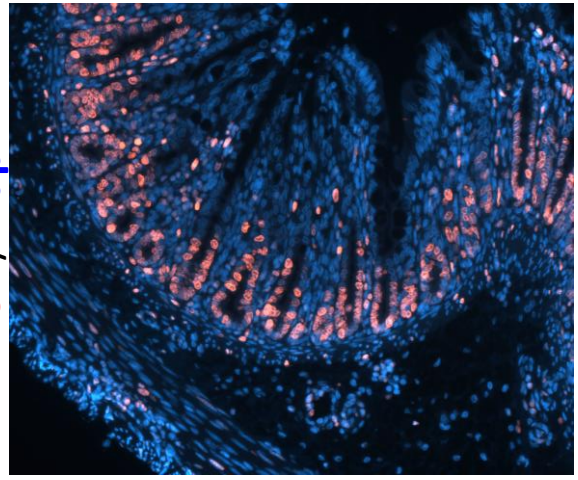
Example of Over Fixation

Tissue can not be in 10% formalin for long term in formalin is OK for material going to routine histological staining (e.g., H&E). Cross-linking action continues and may irreversibly change the conformation (i.e., reduce the antigenicity) of antibody targets.

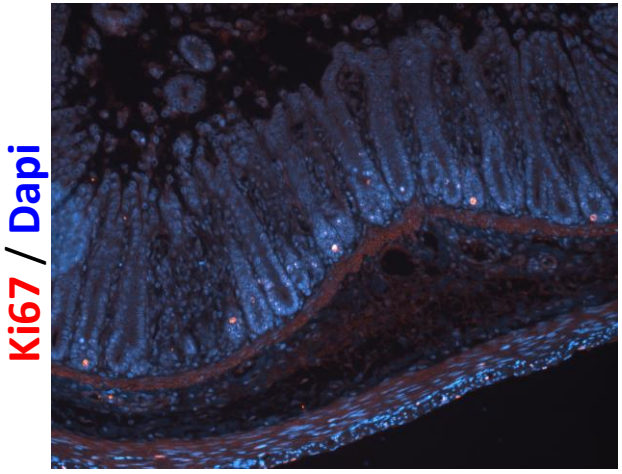
*** Not good if your slides are used for immunostaining.

*** Significantly altering fixation times between batches of samples will have major consequences on the quality of immunostaining.

Normal fixed tissue



Over-fixed tissue



Staining done at the same time but on 2 different groups of mice. Not same fixation condition and incubation.

Over-fixation effects are **antigen depend**. Samples are not good for Ki67 antigens (right picture) but are still good for Claudin3, Claudin2 and UEA staining.

Other fixatives

Chemicals Fixatives : Fixation instantly by a coagulative / precipitating fix process.

Carnoy's: Ethanol: chloroform: acetic acid (60:30:10 v/v)

Methacarn: Same as Carnoy's but with Methanol.
Methanol : chloroform : acetic acid (60:30:10 v/v)

Bouins:
Saturated aqueous picric acid : formalin : acetic acid (15:5:1 v/v)
Use for **Masson Trichrome Staining** (MTS) to visualize collagen.

Carnoy's : Matsuo et al., 1997.

Other fixatives

Pros :

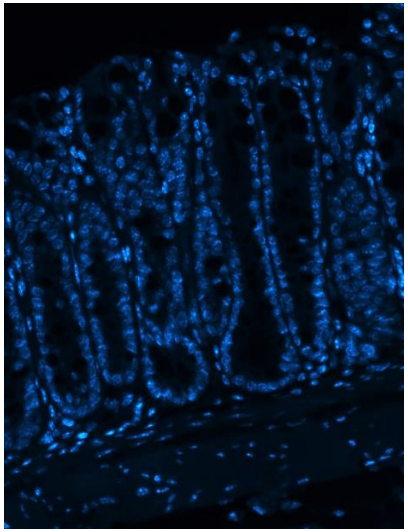
may be useful for collagens, actin, keratins, gfap, **Mucus, Paneth cells, vesicles** conservation.

Cons :

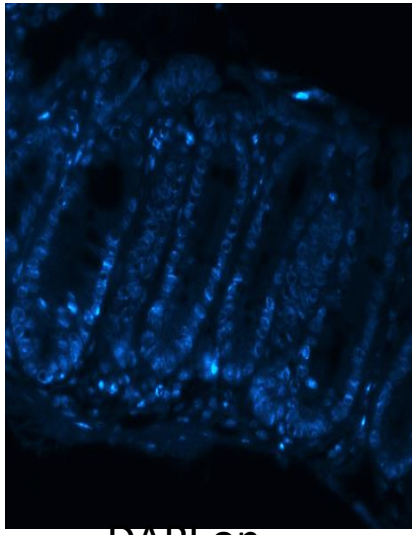
Normal stain (such as H&E, and DAPI) are affected.

Nucleus are too fixed (empty nucleus).

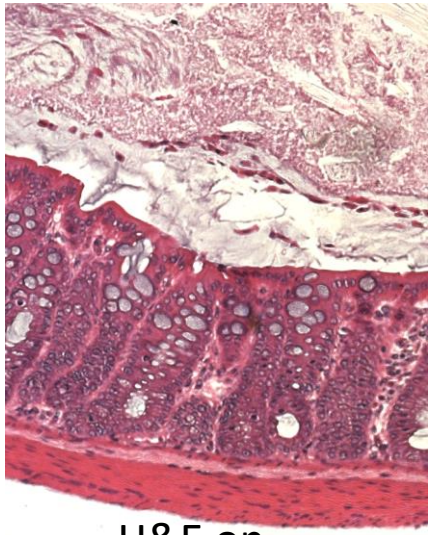
Fixative difficult to remove (extreme rinse)



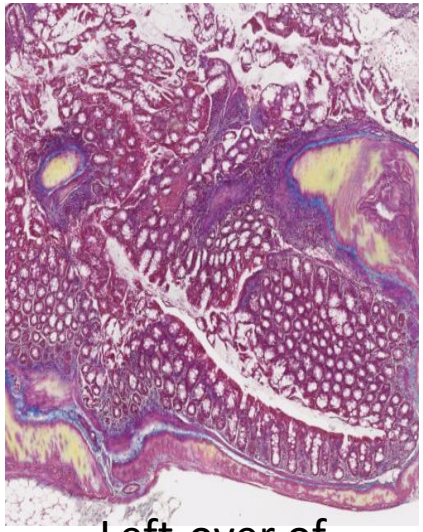
DAPI on Formalin slide



DAPI on Carnoy's slide



H&E on Carnoy's slide



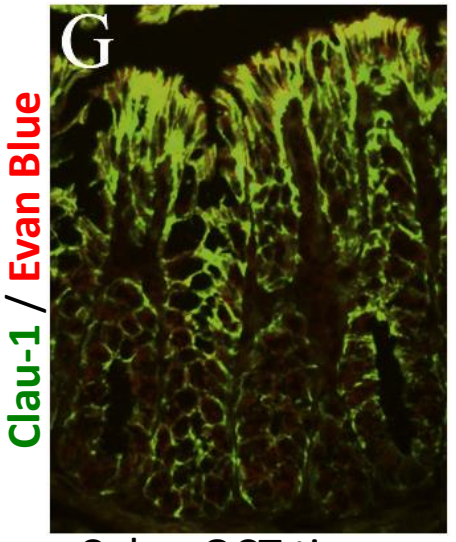
Left-over of Bouin solution

Matsuo et al., 1997.

Other Embedding - Cryopreservation (O.C.T.)

Pros:

- minimal alteration of the protein structures
- **Better staining** for some antibodies
- Avoid / **reduce** the 'auto fluorescence' (i.e., high background)
- the preservation of '**reporter' enzymes** (e.g., beta gal, GFP) that are easily destroyed by fixation
- to do a lipid stain (e.g., Oil Red O)



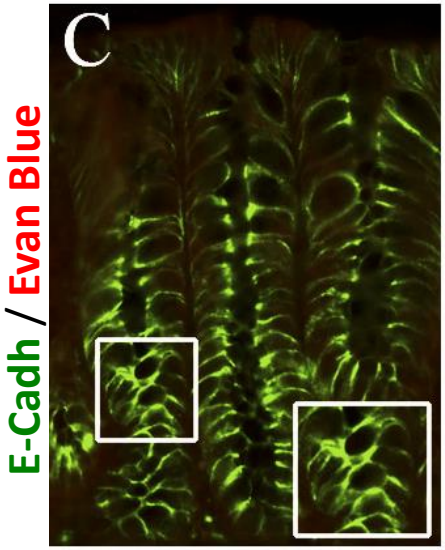
Colon OCT tissue

Cons:

- The morphology of cryo sections is inferior to paraffin sections.

Procedure

Tissues are **snap-frozen in O.C.T.**
Tissue stored at -80°C until ready for sectioning.
Slides stored at **-80°C or -20°C.**
Frozen slides are warmed to RT for **30 minutes.**
To avoid water condensation on slide.
Post-fixed step prior to staining.



Colon FFPE tissue

Histology Procedures

Harvest Fixation (O/N) — Day 1

Rinse and cassette — Day 2

Send work order — Day 2

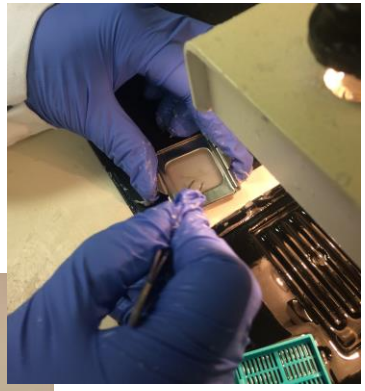
Tissue processing (O/N) — Day 2-3 (or more depending when you sent order)

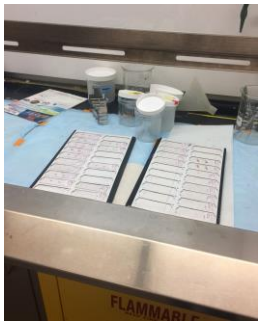
Tissue embedding — Day 3

Sectioning and Dry (O/N) — Day 3-4 (Normally from 1 to 5 hours per order)

H&E and Dry (O/N) — Day 4

FINISH — Day 5





O/N : Over Night incubation

Paraffin processing

Action of tissue dehydration through a series of graded ethanol baths to displace the water, and then infiltration of the tissue with wax (Paraffin).



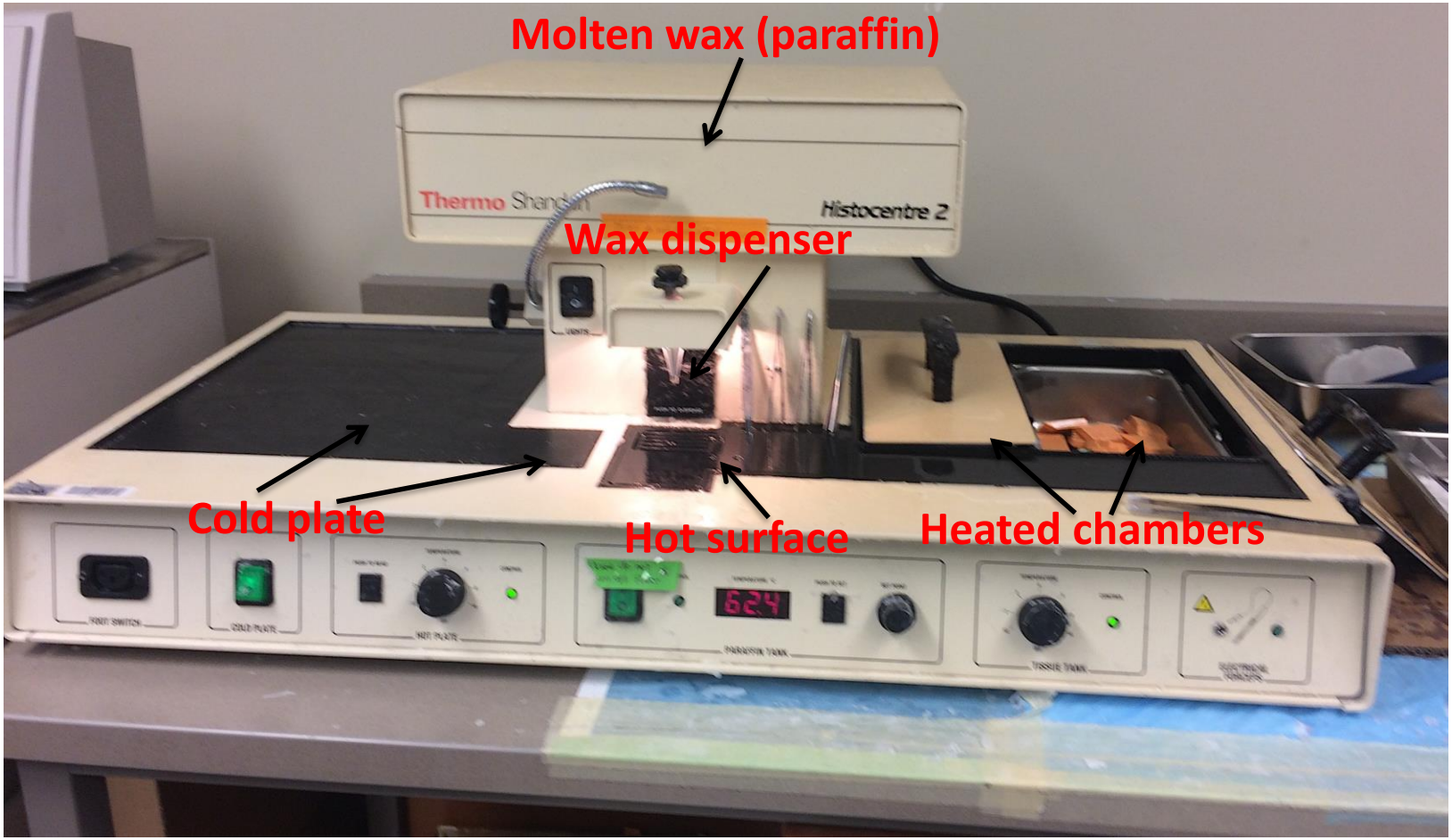

Problems or issues that can occur

Mixing samples from all order together.

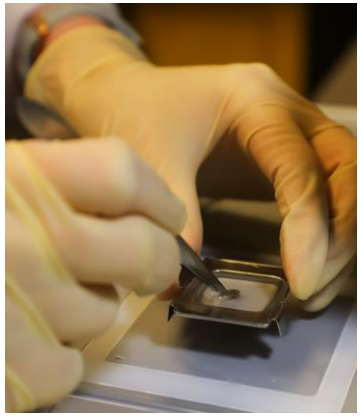
Assure to have distinct name if you have order similar

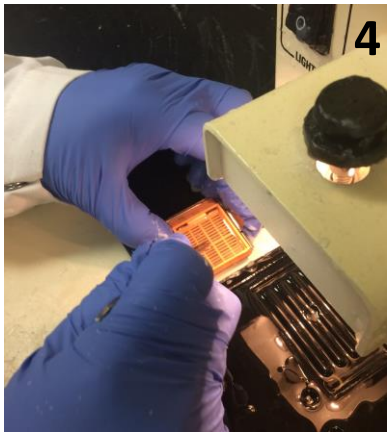
Example : 2 batch of Germfree mice with same treatment
Try to have a specificity for each batch (A, B or C)

Embedding Station

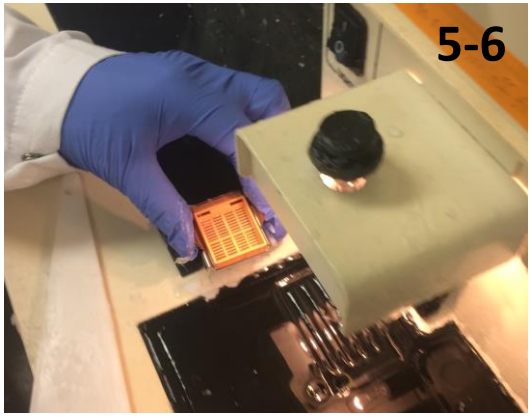


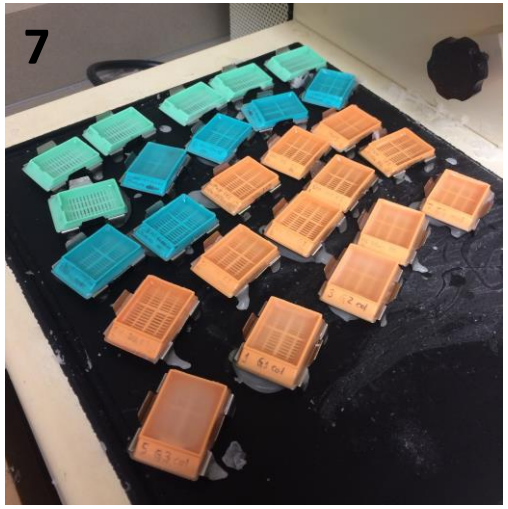
Embedding process

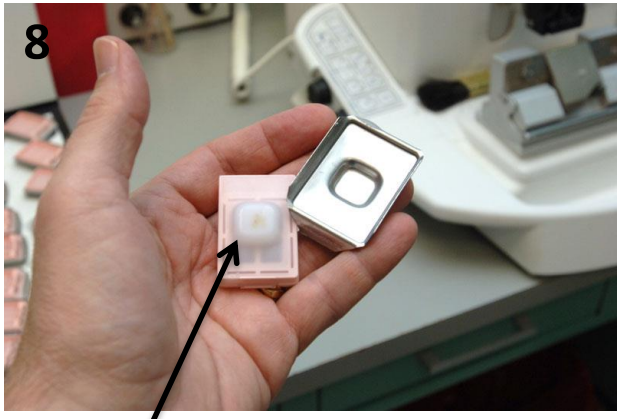




1- Dispense Wax
2- Align Tissue
3- Cool in place
4- Cassette on
5- Top-up wax
6- Cool Plate
7 – Leave to set
8- Unmold

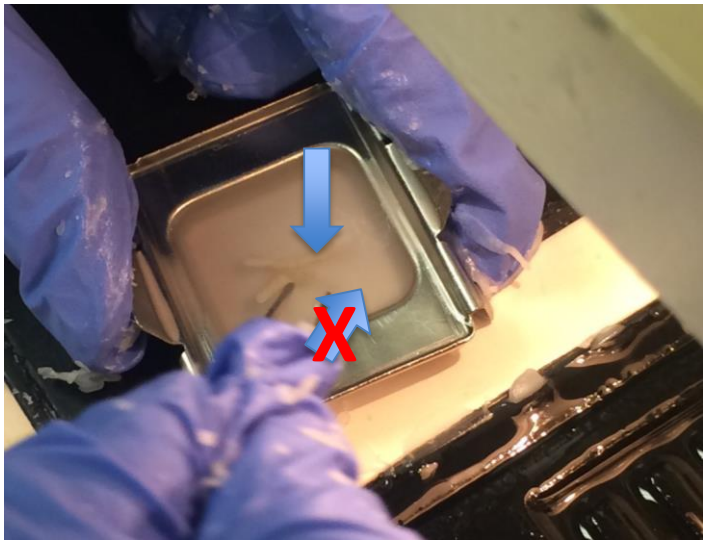


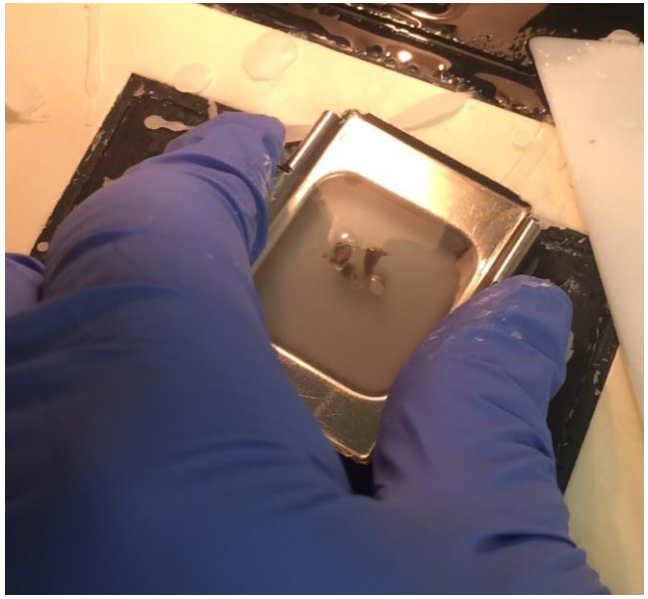




Start to cut from the bottom part of the block

How to optimize embedding ?





1. **Good preparation of your tissue.**

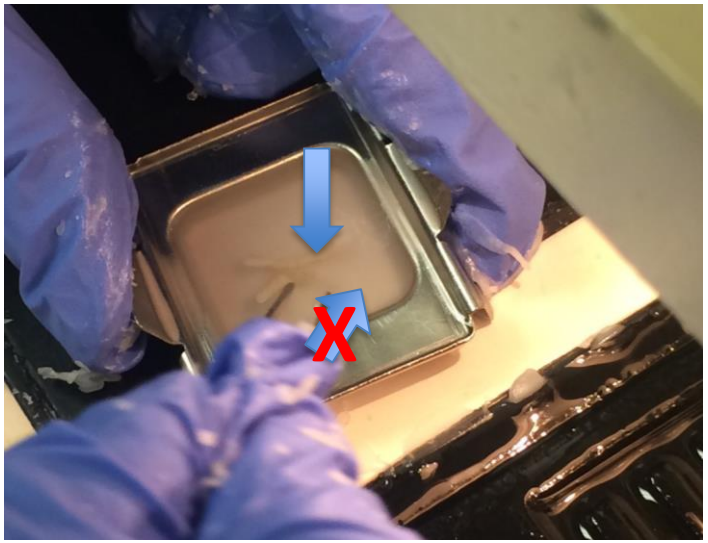
2. **Size your tissue to fit mold** to prevent bad embedding

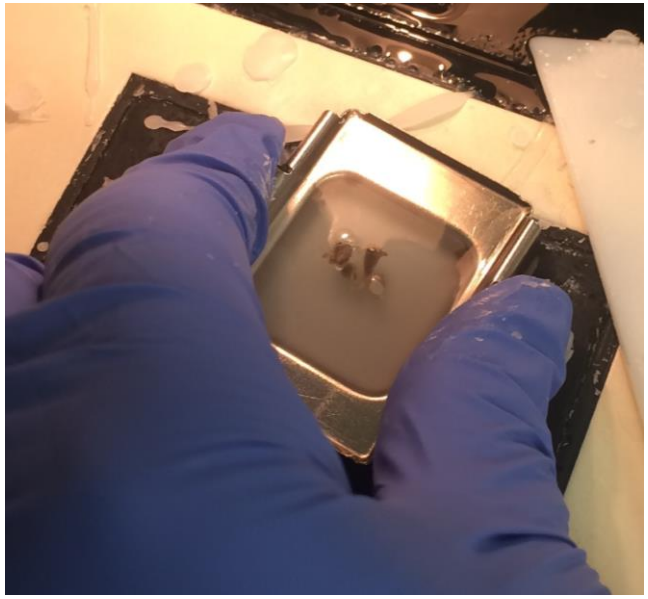
3. **Give instructions** for tissue orientation with illustrations

4. **Place** your sample **in the orientation** you would like it embedded, or **provide instructions** when placing your work order as to how you would like your samples to be embedded.

5. If special instruction/tissue, write your name, extension and precise to call you when embedding

Best Practices





Histology specific instruction

Trim the tissue to fit the cassette and that:

- **does not exceed 4mm** in height
- the maximum size **of 15 x 15 mm** (roughly one third of cassette area).

a. Small tissue (<2mm in height): **only one piece** of tissue per cassette.
b. Any type of gut: **no more than 3 pieces** of tissue per cassette.
c. Gel embedded: **only one piece** of tissue per cassette.
d. For cross sections, ensure that you have cut a **flat edge** which will then be **embedded down.**
e. Only **one type of tissue** per cassette.

**Place your sample in the orientation you would like it embedded, or provide instructions for embedding with your name and extension to call you back.

Labelling your cassettes

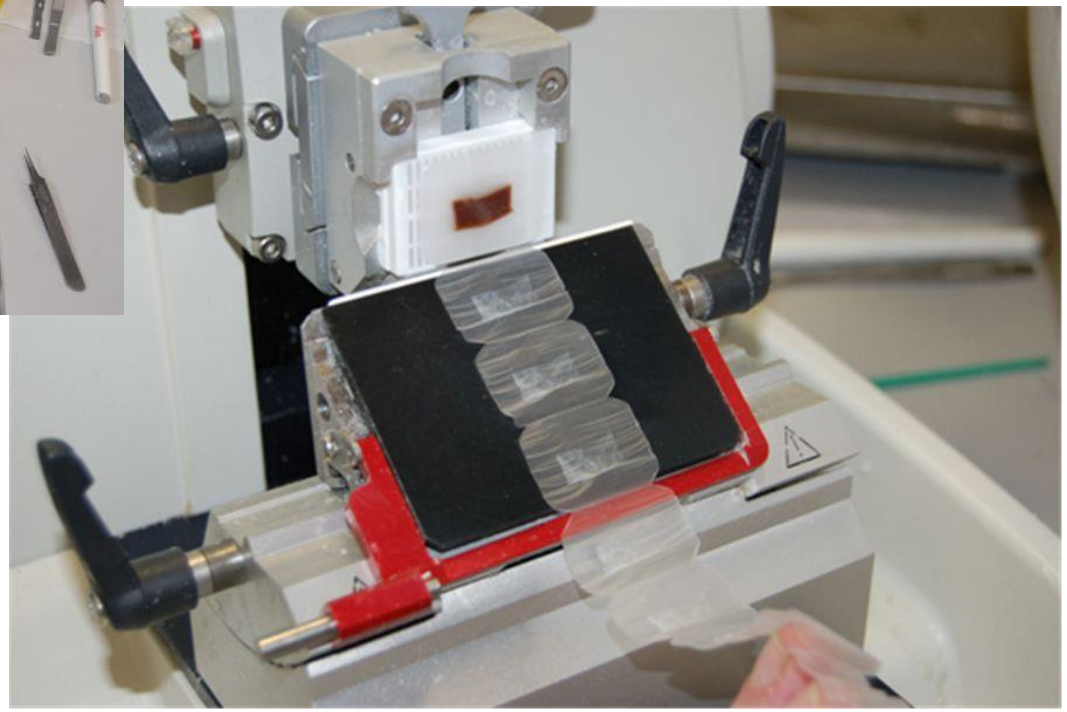
- Label cassettes with a **numerical ID** and a **specific sample ID**, on the front and/or side, with pencil (HB lead or harder). **Numerical ID** begins from 1 to n and corresponds to the number of cassettes (n) that you will submit. The **specific sample ID** can be the experimental ID, treatment, or anything else specific to your samples to differentiate your samples from other projects.
  Example: 1-16D KO col, 2-16D WT col, 3-8D KO col, etc.

  Make sure that the sample ID on the cassette will match the slide IDs on the work order. **You will be contacted if the ID on the work order does not match the cassette.**

Cutting slide

Microtome

1- Trim the block to good sections (Ex : complete circular muscularis layer for gut donut)
2- cut slides
3- Dry (O/N at 50°C) to let section adhere to the slides



** Give **instructions** on where to start sectioning (Prefer all tissues complete or first tissue)

Histology SOP

What can go wrong with your tissue at Histology

Mis-labeling Lost tissue

Bad embedding = Bad cut = Bad area of staining

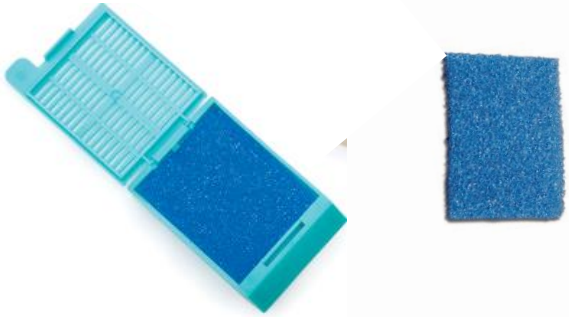
When you should care about your tissue

All steps of your tissue processing should be important for you

Tissue harvest (Fast, cold, Cutting with straight edge) = perfect cut

Instruction for embedding

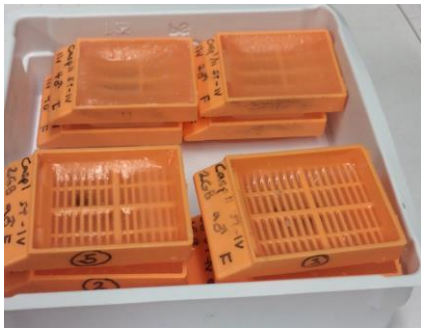
Instruction for cutting / sectioning



Sample preparation :

Trim the tissue to match the tissue cassette* (1X.0.5X0.5cm).

ALWAYS use a biopsy form pad* for any tissue sample that is less than half the volume of the tissue cassette.



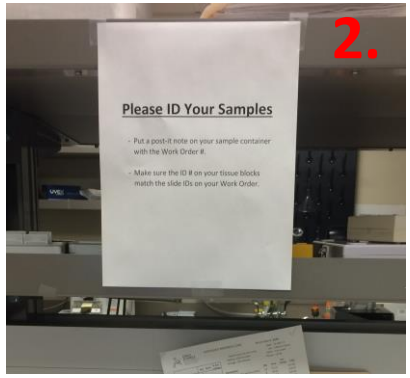
Double check that the cassette is **sealed**

Label cassette with an **indelible marker or a pencil HB**.

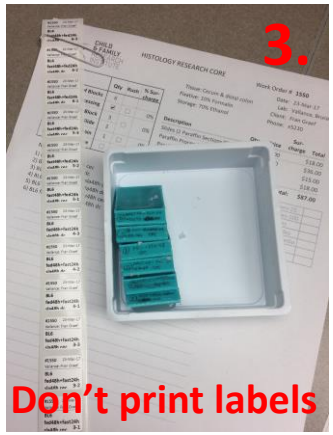
Do not use a “Sharpie”, which is soluble in the organic solvents which are used for processing.

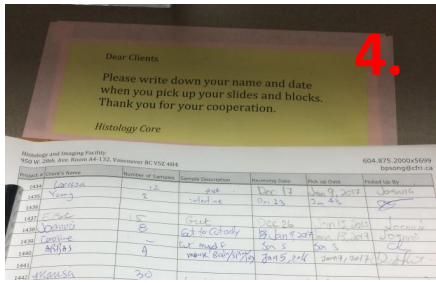
Histology SOP – Samples drop-off

Sample submission checklist:



1. **Bring your fixed tissue** in 70% ethanol (or 100% ethanol in case of Carnoy's fixation) **to the Histology Lab 4**°C refrigerator outside of Histology Lab latest by **Thursday, 2:00pm**.
2. each **cassette numbered numerically from 1 to n**, and include a unique project identifier on each cassette to distinguish your project from another. Example: 1-16D KO col, 2-16D WT col, etc.
3. **Fill out an electronic work order** with all the details at the Histology Lab computer. Make sure that you list all sample IDs in the same format as written on the cassettes. **Print out the work order** and **add the order # on** your container in the fridge. **Please do not print the labels.**
4. **Fill information on the submitting and receiving list** (your name, date, number of samples, and the additional information) at the same number line as your work order number.
5. **For non-standard** processing requirements, **give instructions** (specific embedding orientation, or any other special requests, please write down or illustrate all the details) and **check with a histology lab staff** that the requirements are understood.
6. **For a gel embedded tissue** (ex. Organoids, islets, etc.), **please speak with a histology lab staff** when submitting your work order so that we can better understand how to best work with your tissues to suit your needs.

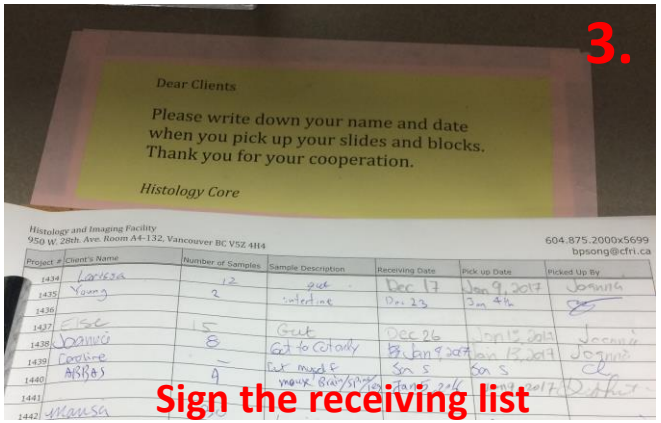




Histology SOP– Samples pickup

Sample pickup checklist:

1. **You will get an email notification** when your slides/samples are ready for pickup. Please do not pick up anything until you receive the email.
2. When you get notified about your order completion, please **pick up your slides and blocks** from the pickup shelf above the benchtop. Please **do not remove your work order** sheet.
3. **Sign** your name and the date of the pickup on the submitting and receiving list when you pick up any slides/samples.
4. Take away your container from the box by the door.



Take home message

Understand each step of the histological workflow. Careful planning and execution of the steps described in this presentation will yield optimal histopathological preservation.

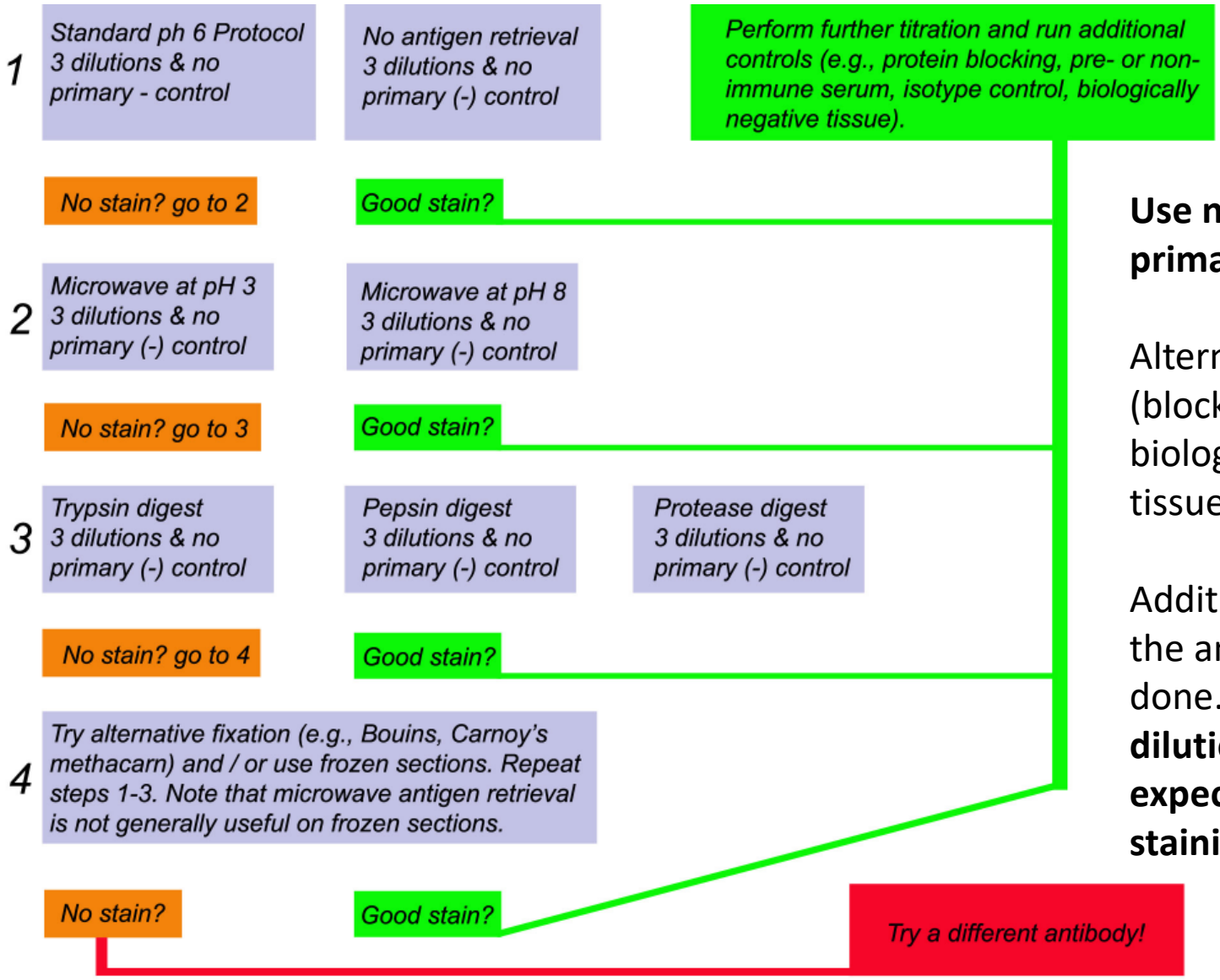
Tissue collection and **preparation** is very important

Consistent in fixation conditions to reduce variation in staining results.

Instructions, Instructions, Instructions for Histology staff **!!!!!**

Clear label with **numbers and unique naming**

Troubleshooting IHC



Use negative control (no primary Ab)

Alternative control (blocking peptide, biologically negative tissue)

Additional titration of the antibody is also done. **(test other dilution to get expected/published staining)**