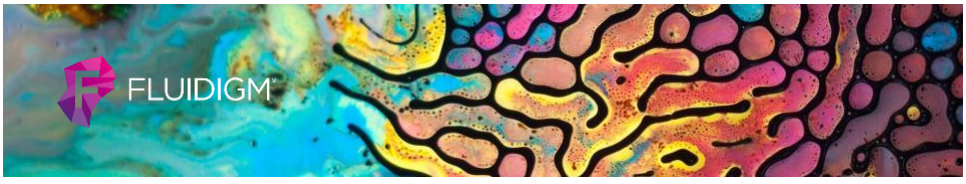
Hyperion Imaging System

Specimen preparation



Learning objectives

Understand the basic principles of FFPE tissue processing and how this affects slide processing

Understand best practices for frozen tissue processing

Be able to perform staining of a single slide

Understand why titration of antibodies is important

FFPE tissue processing

FFPE tissue processing

Formalin fixation and paraffin wax embedding (FFPE) is the gold standard for histopathology.

Processing fresh tissue into slides involves the following steps:

1. Fixation of tissue
2. Dehydration and clearing of tissue
3. Embedding in paraffin wax
4. Sectioning of slides

Fixation of tissue

Fixatives are split into different groups depending on their chemical interaction with tissues.

Fixative Group	Method of Action
Aldehydes	Create cross-links between lysine residues
Coagulants	The alcohols; cause coagulation of proteins
Acids	Also called “additive coagulants;” cause coagulation of proteins by direct interaction
Mercurials	No longer used, but archival tissue still exists
Mixed fixatives	A mixture of the groups listed above

Fixation of tissue

Cross-Linking	Coagulants	Acidic Coagulants
Formaldehyde	Ethanol	Picric acid
Glutaraldehyde	Methanol	Acetic acid
Mixed Fixatives		
Carnoy’s fixative	Methacarn	Zinc formalin
Bouin’s solution	Formol acetic alcohol	Zenker’s fixative

Fixation of tissue

Main points

All fixatives have different methods of action.

Some fixatives contain metals that may be detectable during ablation.

Formalin fixation creates cross-links between molecules, which can mask over epitopes. These epitopes need to be unmasked and retrieved during slide staining.

Fluidigm FFPE antibodies are validated in 10% NBF-fixed tissues and are not guaranteed to work for other fixation methods.

Suspension antibodies may not work in fixed tissues.

Dehydration of tissue

Purpose
Dehydrate tissue by removal of water and replacement with alcohol

Method
Submerging tissue in an alcohol such as:

- Ethanol
- Methanol
- Industrial methylated spirits

Alcohol is a fixative. Do not store tissues in alcohol for extended periods (more than 2–3 days).

Clearing of tissue

Purpose
Remove alcohol from tissue and replace with a paraffin wax-miscible hydrocarbon

Method
Submerging tissue in aromatic hydrocarbon:
- Xylene
- Toluene
- Commercial alternatives: Clearene, Histo-Clear®, etc.

Embedding and sectioning

Purpose
Remove clearing agent from tissue and embed in wax for sectioning and long-term block storage

Method
- Tissue is submerged in molten wax under pressure to replace clearing agent.
- Tissue is embedded in a wax mold and cooled to room temperature.
- Sections are cut with a microtome.
- Sections are stained.
- Residual tissue block is put into storage.

Embedding and sectioning
Main points

Antigenicity of both “new” and sectioned FFPE blocks is stable for many years.

In comparison, antigenicity of tissue sections degrades over several weeks.

Freshly cut sections (no older than 2–3 weeks) should be used, where possible.

Phosphorylated targets are very sensitive to degradation. Sections should be no older than 1–2 weeks.

Slide preparation

Staining protocol summary

1. Bake slides 1 hour at 60 °C.
2. Dewax in xylene.
3. Rehydrate in descending ethanol grades (100%, 95%, 80%, 70%).
4. ddH2O and PBS washes
5. Basic pH antigen retrieval at 95 °C
6. Block with 3% BSA in PBS.
7. Incubate in primary antibodies.
8. Intercalator-Ir in PBS
9. ddH2O rinse
10. Air-dry.

1–4. Rehydration of tissue

The first three steps of the protocol undo the dehydration and clearing that is performed during tissue processing.

- Bake slides for 1 hour at 60 °C after sectioning.
  Adheres the section to the slide
- Soak in a Coplin jar of xylene for 20 minutes
  Removes the wax from the section
- 5 minute soak in descending ethanol grades (100%, 95%, 80%, 70%)
  Slowly rehydrates the tissue
- Rinse in ddH20 for 5 minutes.
  Rinses residual ethanol from the slides

5. Basic pH antigen retrieval

Antigen retrieval breaks the cross-links that form during tissue fixation, unmasking the antigens for the antibodies to bind to.

- Fill 50 mL centrifuge tube with antigen retrieval solution.
- Insert into heating block and preheat to 95 °C.
- Insert slides and incubate for 30 minutes at 95 °C.
- Cool to room temperature (RT), approximately 20–30 minutes.
- Rinse the slide with ddH2O for 10 minutes.
- Wash the slide with DPBS for 10 minutes.

6. Blocking with BSA

Blocking steps help to increase the specificity and sensitivity of the stain by blocking nonspecific binding of antibodies.

- Use PAP pen to circle the sample.
  Reduces the volume of reagents needed
- Block with 3% BSA in DPBS for 45 minutes at RT.
  Inhibits nonspecific binding of antibody

7. Antibody staining

- Centrifuge antibodies for 2 minutes.
- Calculate the total volume of antibody cocktail needed.
- Calculate the amounts of antibody and diluent (0.5% BSA in DPBS) needed for the total volume.
- Cocktail should be created by adding primary antibody to the diluent.
- Mix/vortex the antibody cocktail thoroughly.
- Pipette antibody cocktail onto slide and incubate overnight at 4 °C in a humidity chamber.

8. Intercalator staining

- Wash in 0.1% Triton™ X in DPBS for 8 minutes with gentle agitation. Repeat twice.
- Stain the tissue with Intercalator-Ir (1:400) in DPBS for 30 minutes at RT.
- Rinse in ddH2O for 5 minutes.
- Air-dry the slide for at least 20 minutes at RT.

Frozen tissue processing

Frozen tissue processing

FFPE tissue

1. Fixation of tissue
2. Dehydration and clearing of tissue
3. Embedding in paraffin wax
4. Sectioning of slides

Frozen tissue

1. Snap-freeze in isopropanol
2. Sectioning of slides

Frozen staining protocol

1. Dewax in xylene.
2. Hydrate in descending ethanol grades (100%, 95%, 80%, 70%).
3. ddH_2O and PBS washes
4. Basic pH antigen retrieval at 95 °C
5. Block with superblock*
6. Incubate in primary antibodies
7. Intercalator-Ir in PBS
8. ddH_2O rinse, 5 minutes
9. Air-dry.

* Different from blocking for formalin-fixed sections, in which 3% BSA is used

Gray = not necessary for frozen sections..

Frozen tissue processing
Main points

1. Frozen tissues do not require dewaxing, rehydration or antigen retrieval.
2. Unstained frozen sections should be stored at –80 °C until needed.
3. Antibodies from the suspension catalogue are likely to work.
4. Phosphorylated targets are very sensitive to degradation. Sections should be no older than 1–2 weeks.

Simplify the complex quest to understand and apply biology.