Part 1 : *Understanding Basic Preanalytics*

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Understanding Basic Preanalytics Learning Objectives

1.

Understand pre-analytic factors

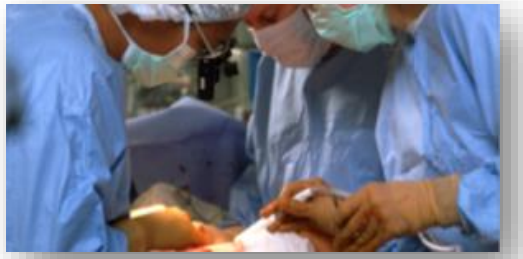
Outline the process of tissue diagnostic workflow in the laboratory

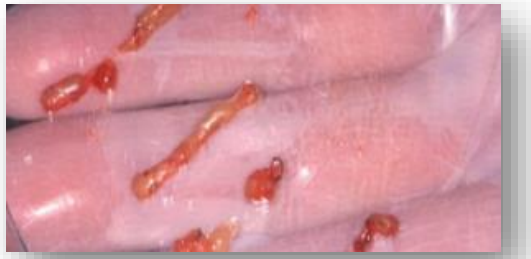
2.

Describe best practices to optimize tissue preservation and quality

Provide information on how to provide standardization and optimize best practices

What happens to the patient sample after procurement during surgery?





Work cell description

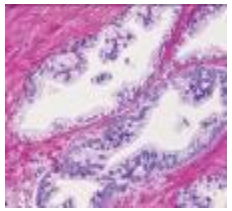
Pre-analytical

Tissue preparation

- Labeling/Requisition
- Tracking
- Grossing
- Fixation
- Processing

Primary staining

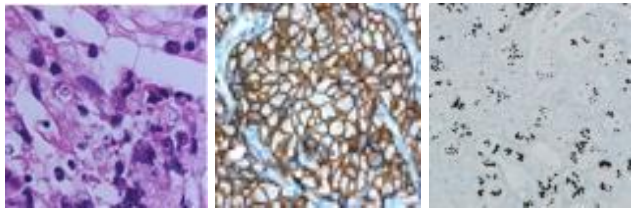
H&E stain



- Binary diagnosis
- Tissue
- Morphology

Advanced staining

SS IHC ISH



- Diagnosis
- Detection of molecular target
- Predictive/prognostic markers

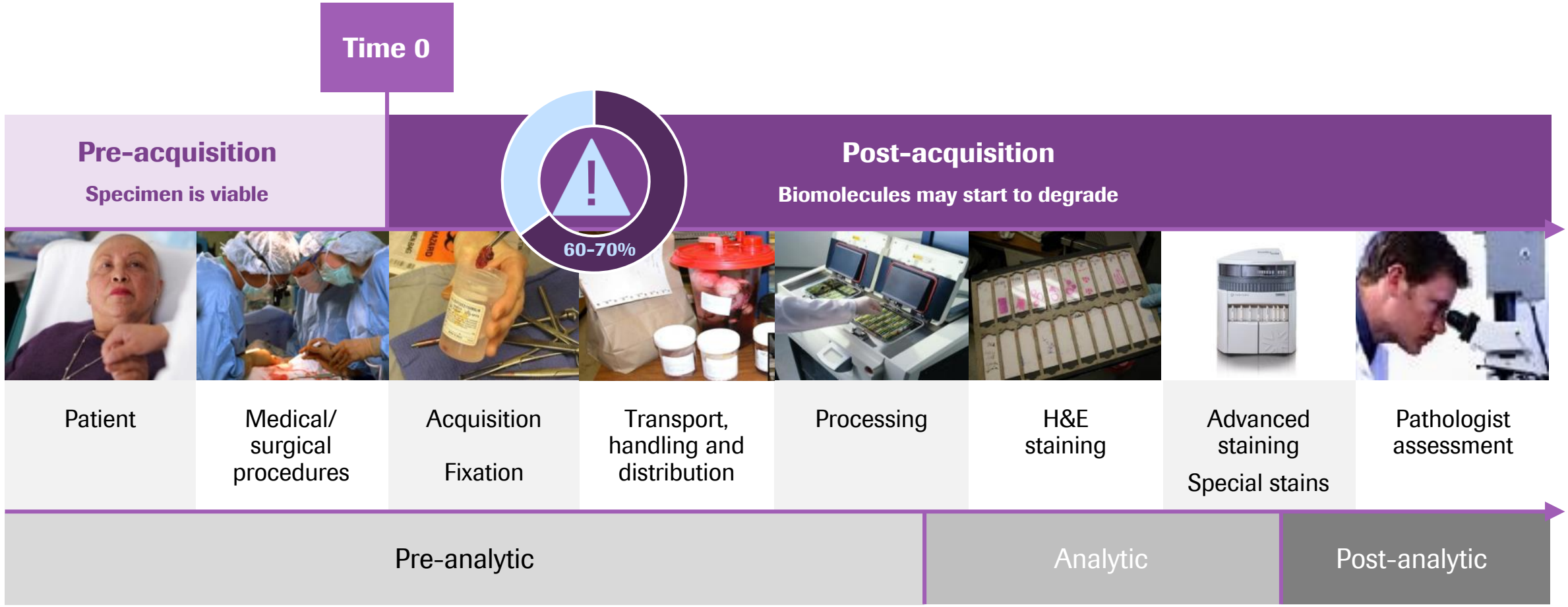
Imaging/reporting

Image analysis

- Quantitative analyses
- Report generation
- Telepathology
- Archiving

The journey of the tissue specimen

Pre-analytic process has many opportunities for human error that can compromise the quality of the diagnosis



Pre-analytic variables

Many sources of potential errors

Sample to transport

- Transfer temperature
- Sample size
- Tissue type
- Transfer conditions
- Fresh vs. Fixed
- Type of container

Reception to fixation

- Time to fixation
- Type of fixative
- Quality of fixative
- Quantity of fixative
- Temperature of fixation
- Tissue grossing techniques

Dehydration to embedding

- Processor
- Protocols
- Bath duration
- Quality of reagents
- Quantity of reagents
- Tissue Orientation
- Temperature of paraffin and time in liquid paraffin

Microtomy to slide

- Section thickness
- Water bath: cleanliness, temp
- Type of slides (coated or plain?)
- Slide drying temp
- Staining (H&E vs. special)
- Mounting (cover-slipping) techniques

Slide drying

- Duration
- Temperature

Archival

- Duration
- Temperature
- Type of support

Many opportunities for variables across pre-analytic pathway to impact downstream testing

| Adapted from Engel K. Arch Pathol Lab Med 2011

Tissue diagnostic workflow

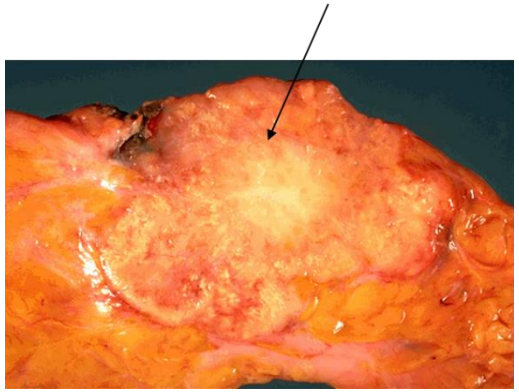
Specimen types *Histology lab*

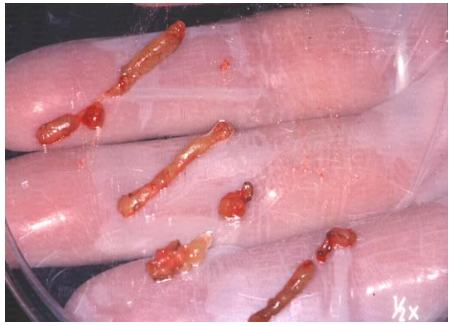
Histology specimens usually consist of pieces of tissue of various methods, and may include:

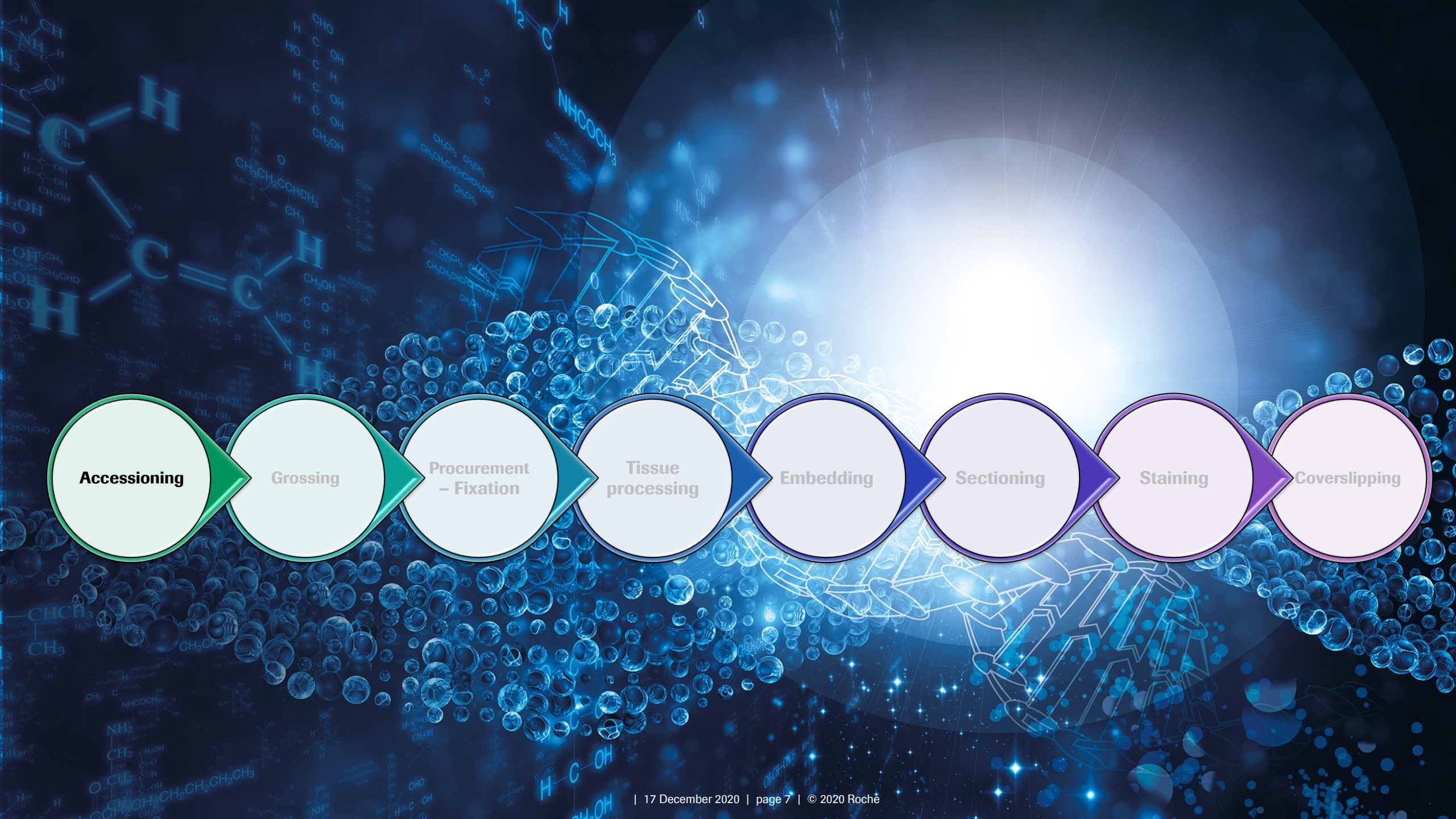
- Small biopsies (such as breast, kidney, prostate, liver, colon, heart or skin)
- Larger specimens removed at surgery (resection specimens)
- Tissues from autopsy

Specimens may be accepted in the Histology Lab from various locations:

- Doctors' offices
- Outside clinics
- Hospital clinics
- Hospital surgeries






Accessioning
Grossing
Procurement – Fixation
Tissue processing
Embedding
Sectioning
Staining
Coverslipping

Accessioning

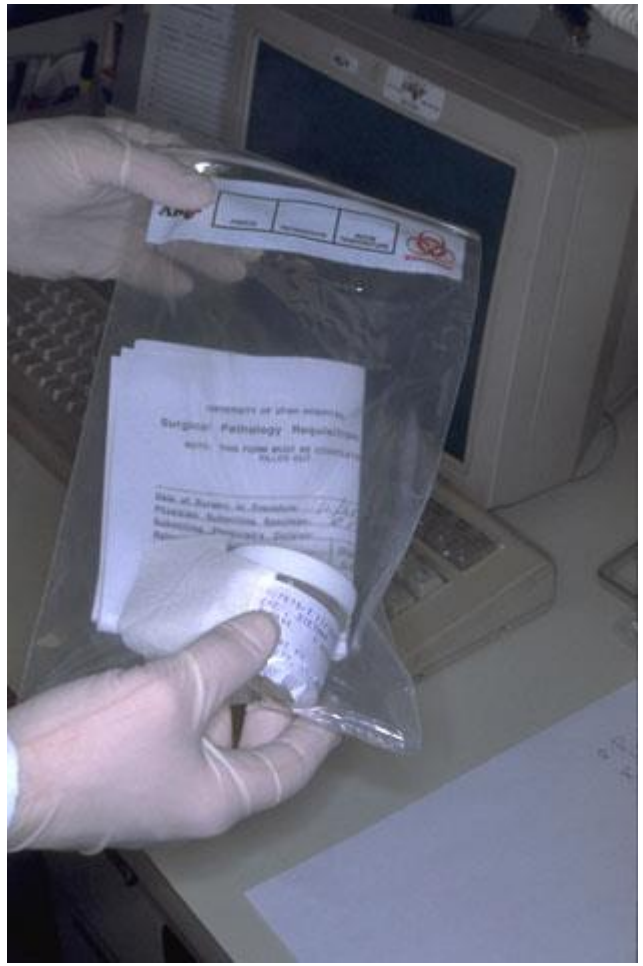
Specimens are received in the histology laboratory

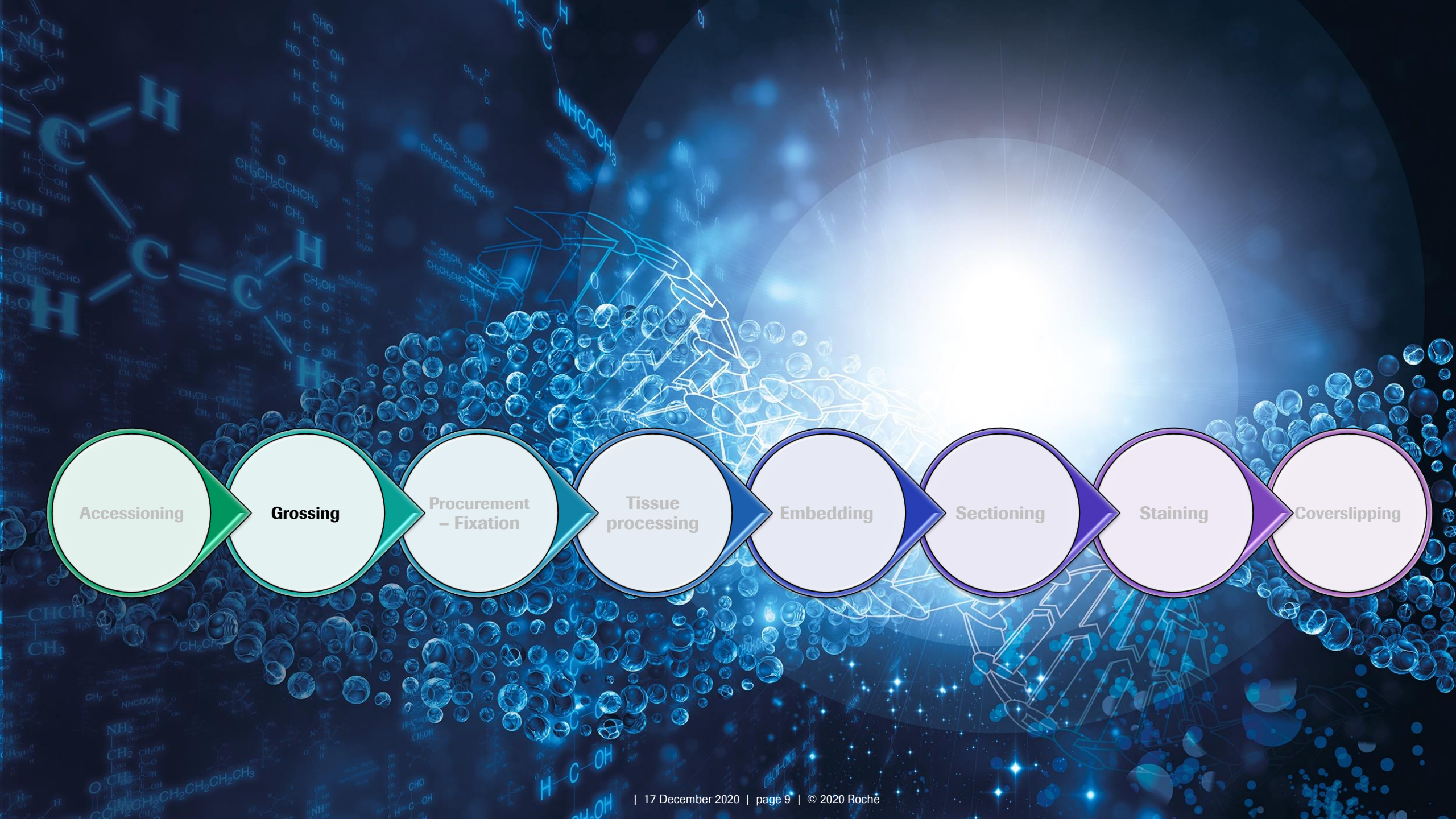
Before gross examination

- Specimens are given a case number
  - Computer system
  - Logbook
- Request sheet and specimen containers are properly labeled
- Cassettes are made

- **Accuracy of all the above is checked**.

This process ‘can be’ a MAJOR source of error in the histology laboratory

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Gross examination

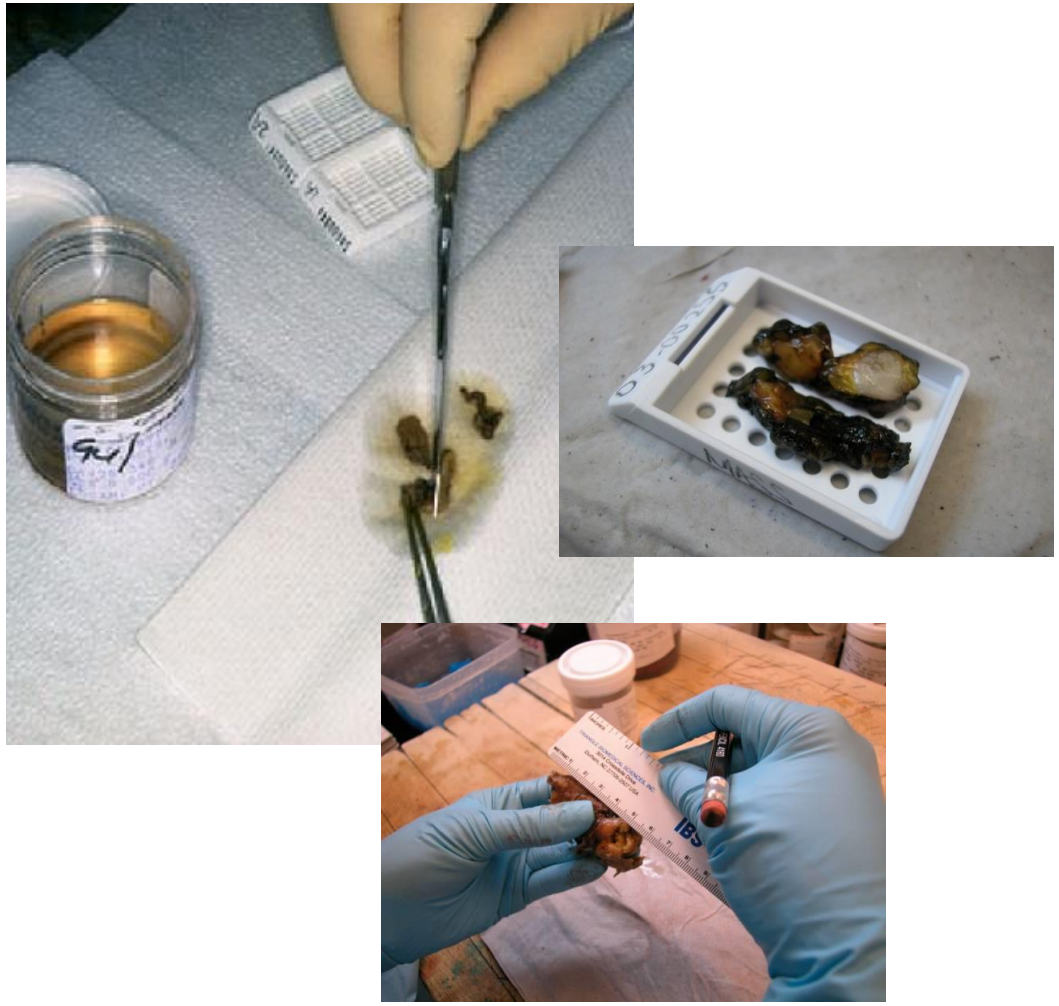
Tissues must then undergo gross examination and dissection

Gross examination or “grossing” consists of:

- Describing the specimen’s size, shape, color and any apparent abnormalities
- Description of margins and their orientation

Depending on the size and type of specimen, it is either submitted entirely
Or a 'representative' section is taken

The tissue is placed into small plastic cassette, which will allow fluids to infiltrate the specimens in the processing step

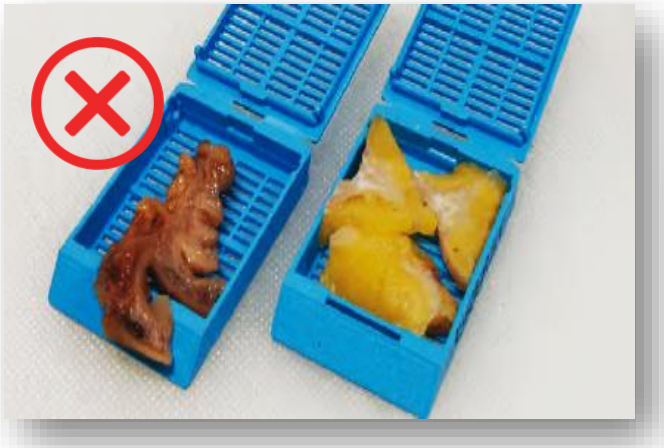


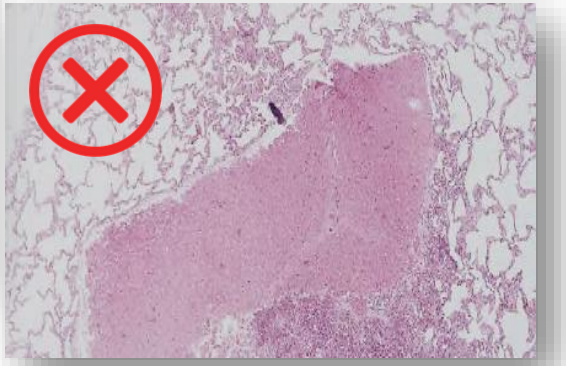
Grossing

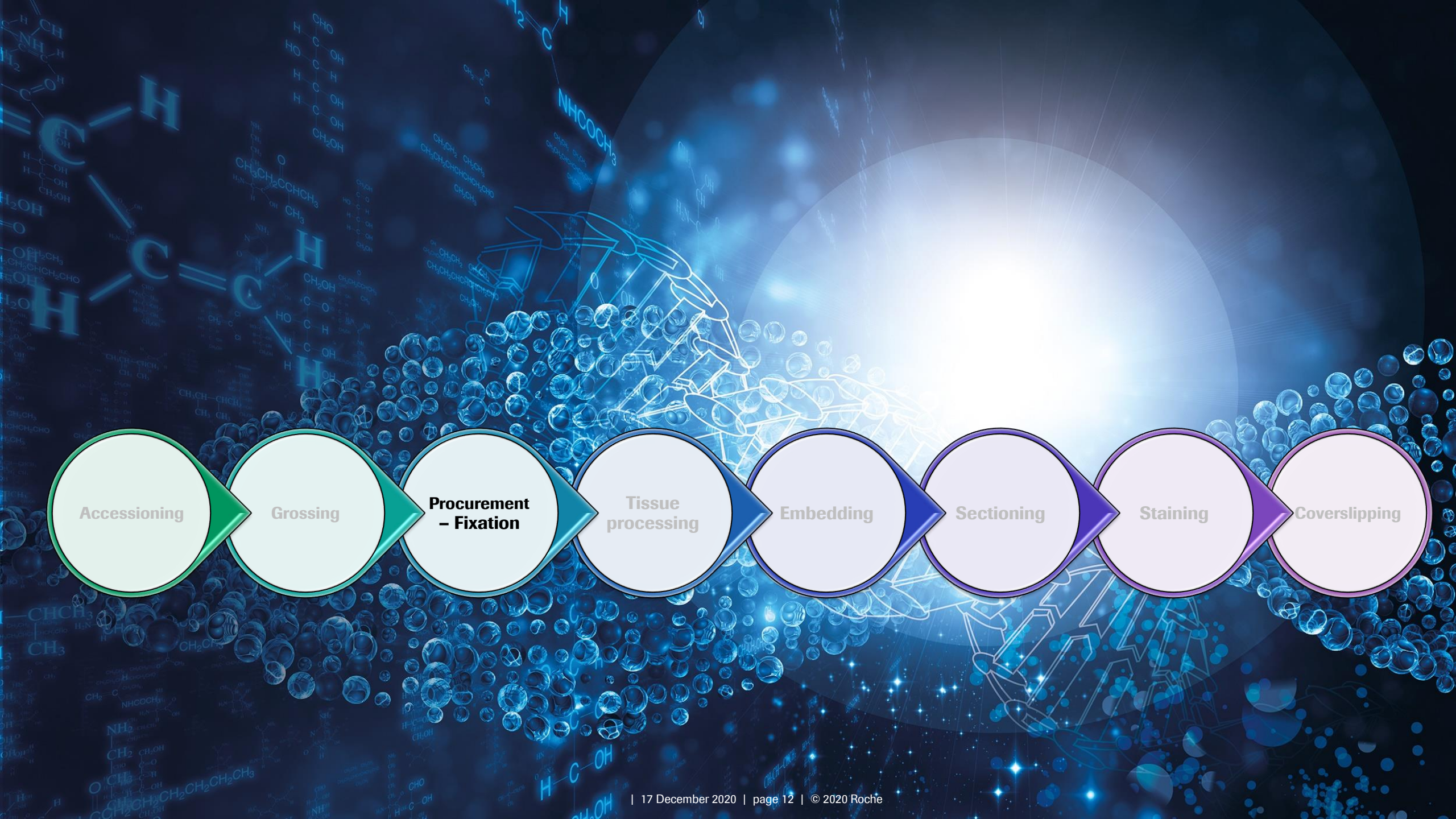
- Check fixation status
- Prepare thin slices 2-3 mm
- Avoid specimen trauma
- Avoid cross-contamination
- Avoid overloading cassettes
- Clearly and properly label cassettes

Recommended Solutions

- For routine processing schedules, grossed tissue sections should be **no more than 3 mm thick**. When processing on a short protocol, the grossed sections must be even thinner or the reagents will not completely penetrate the section.
- At no time should a grossed section be so thick that it touches both the top and bottom of the tissue-processing cassette.



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Fixation

Definition: alters tissue by stabilizing the protein so it is resistant to further changes

10% neutral buffered formalin (NBF) is the most widely used fixative worldwide and is recommended for routine histologic examination and subsequent molecular testing

Fixation is a crucial step in the processing of tissue specimens

- Stabilizes the tissue and stops degradation of macromolecules and loss of potential biomarker information
- Helps preserve tissue and cellular architecture and composition of cells to allow them to withstand processing
- Prevents breakdown of the tissue and molecular features by enzymatic activity and/or microorganisms during long-term storage for future diagnostic testing
- Physico-chemical process that is gradual and complex, requiring sufficient time for diffusion of fixative into the tissue and formation of cross-links

| Thavarajah R et al. J Oral Maxillofac Pathol 2012

Fixation recommendations

ASCO/CAP Guidelines for HER2, ER and PR testing

Fixative: 10% Neutral buffered formalin (NBF)

Time:

- Cold ischemia time: Placed immediately in formalin fixative within 1 hour from time of removal of patient
- Minimum time in fixative: 6 hours (optimal 24 to 48 hours)
- Maximum time in fixative: 72 hours

Temperature:

- Room temperatures (22-25 degrees C)

Volume:

- 15-20:1 ratio of fixative to tissue specimen
- Large specimens should be opened and thinly sliced using proper grossing technique to maximize surface exposure to reagents

| Wolff A et al. J Clin Oncol 2018 | Hammond E et al. Arch Pathol Lab Med 2010 | Allison K et al. Arch Pathol Lab Med 2020 | Carson F. Histologic Fixation and Processing 2009

Fixation

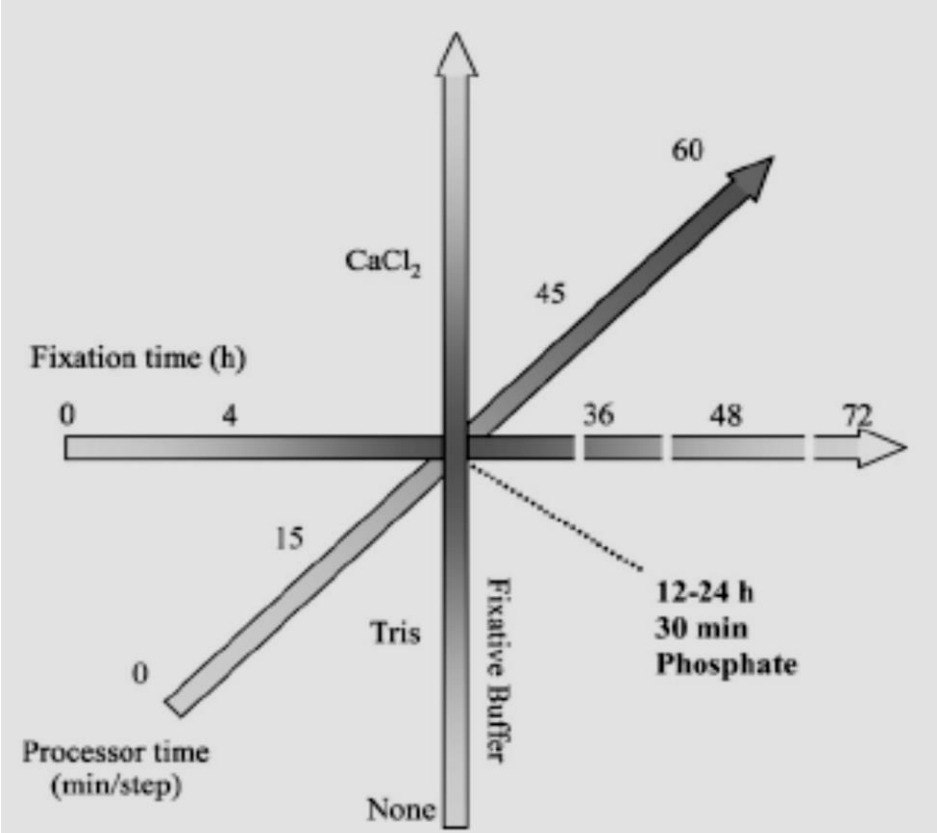
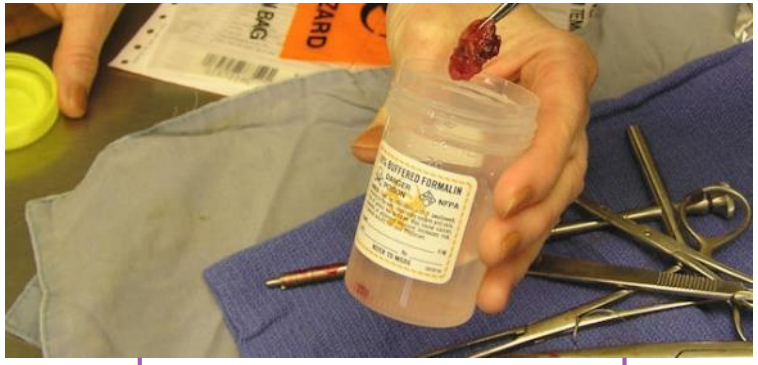


Figure 7 The hypothetical basic model of RNA quality depending on FFPE tissue processing. The darker gray color represents conditions associated with better RNA quality from FFPE tissue.

Chung JY, Braunschweig T, Williams R, Guerrero N, Hoffmann KM, Kwon M, Song YK, Libutti SK, Hewitt SM. Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. J Histochem Cytochem. 2008 Nov;56(11):1033-42.

Fixation definitions



Time TO fixation

Cold ischemic time

Time from the removal of the tissue until placement in fixative

1 hour or less

Time OF fixation

Fixation

Time that tissue specimen is immersed in fixative

At least 6 hours, not more than 24–72 hours

Lott R et al. Practical Guide to Specimen Handling in Surgical Pathology 2018

Hematoxylin and eosin (H&E) stain

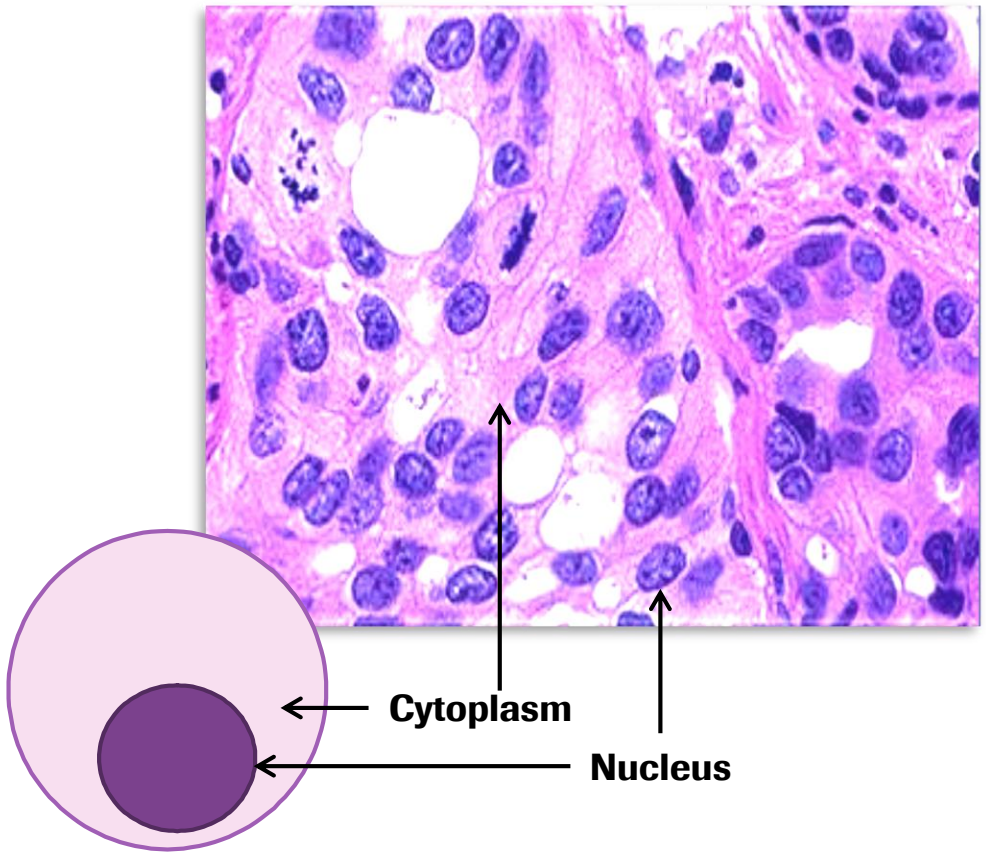
Primary stain used in the diagnostic process in Pathology

Most widely used histologic stain

Two-color process

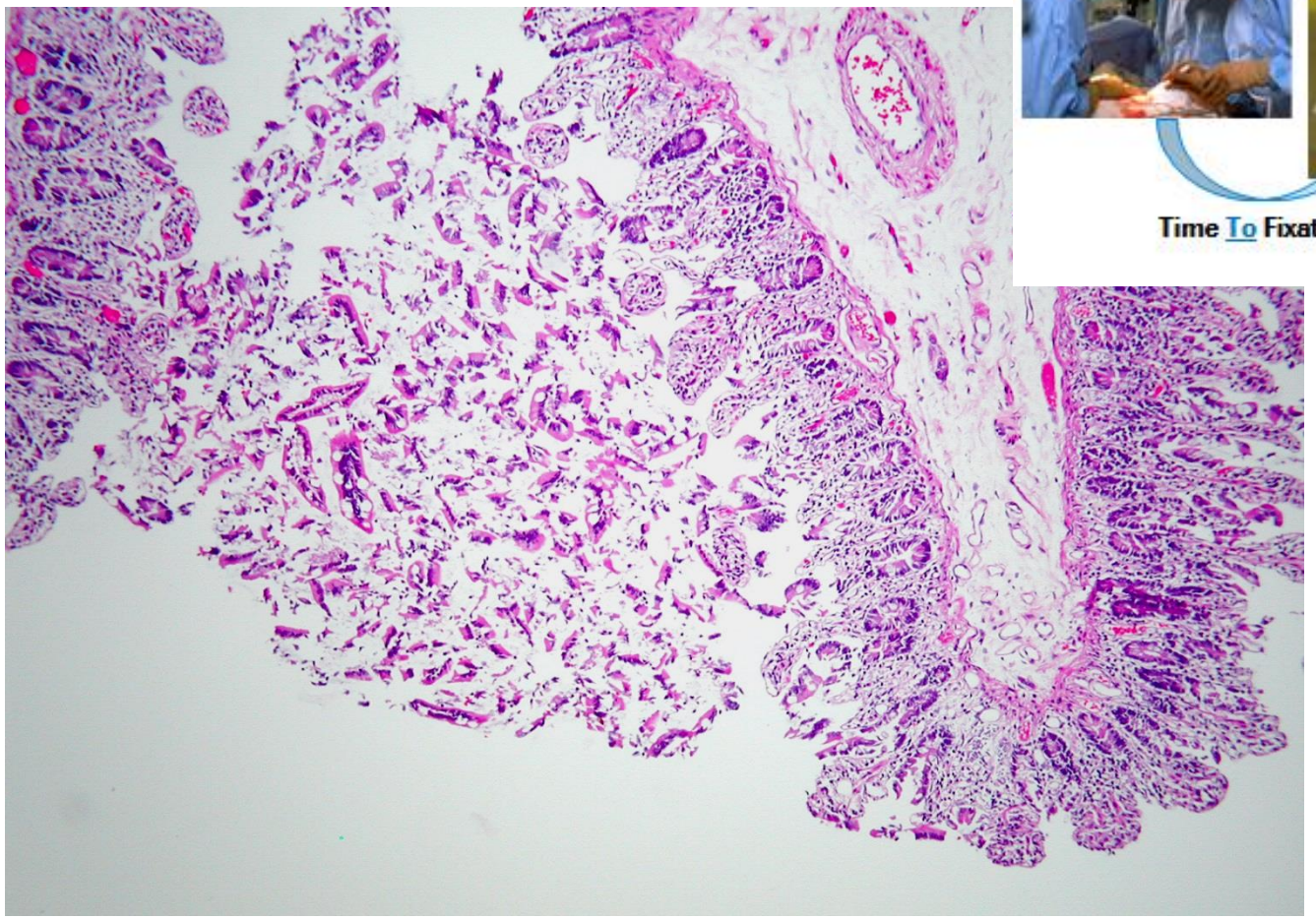
Hematoxylin is a bluish purple
dye which stains the nucleus of cells
- *stains acidic components (nucleic acid)*

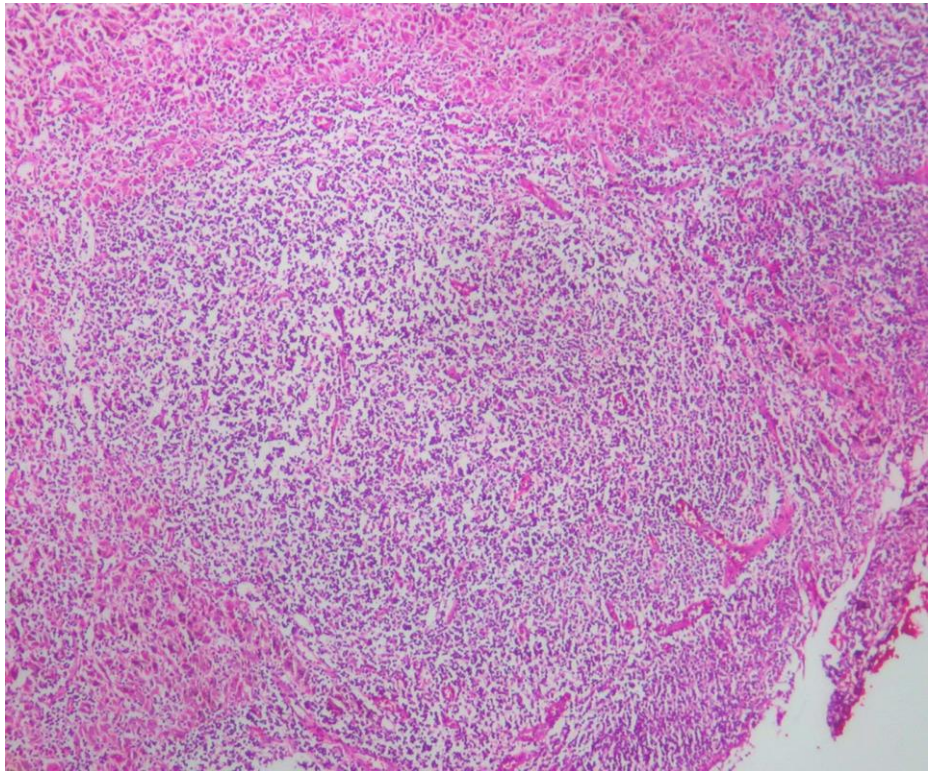
Eosin is a reddish dye and stains the extracellular matrix and cytoplasm of cells pink
- *stains basic components (many proteins)*

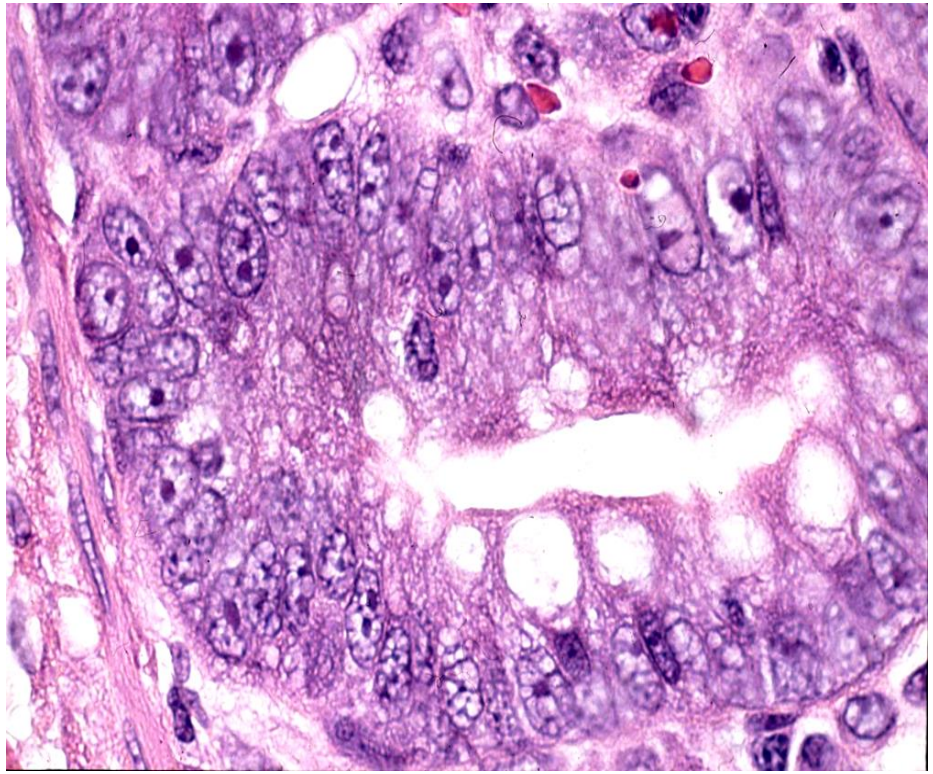


| With kind permission from Robert Lott

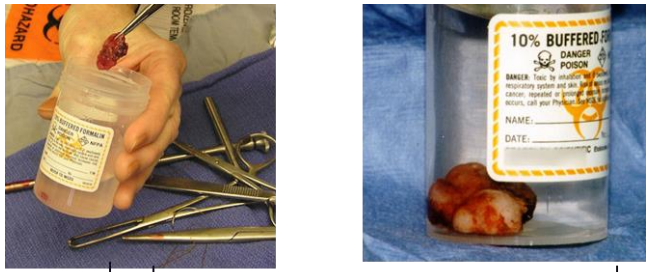
Fixation Delayed





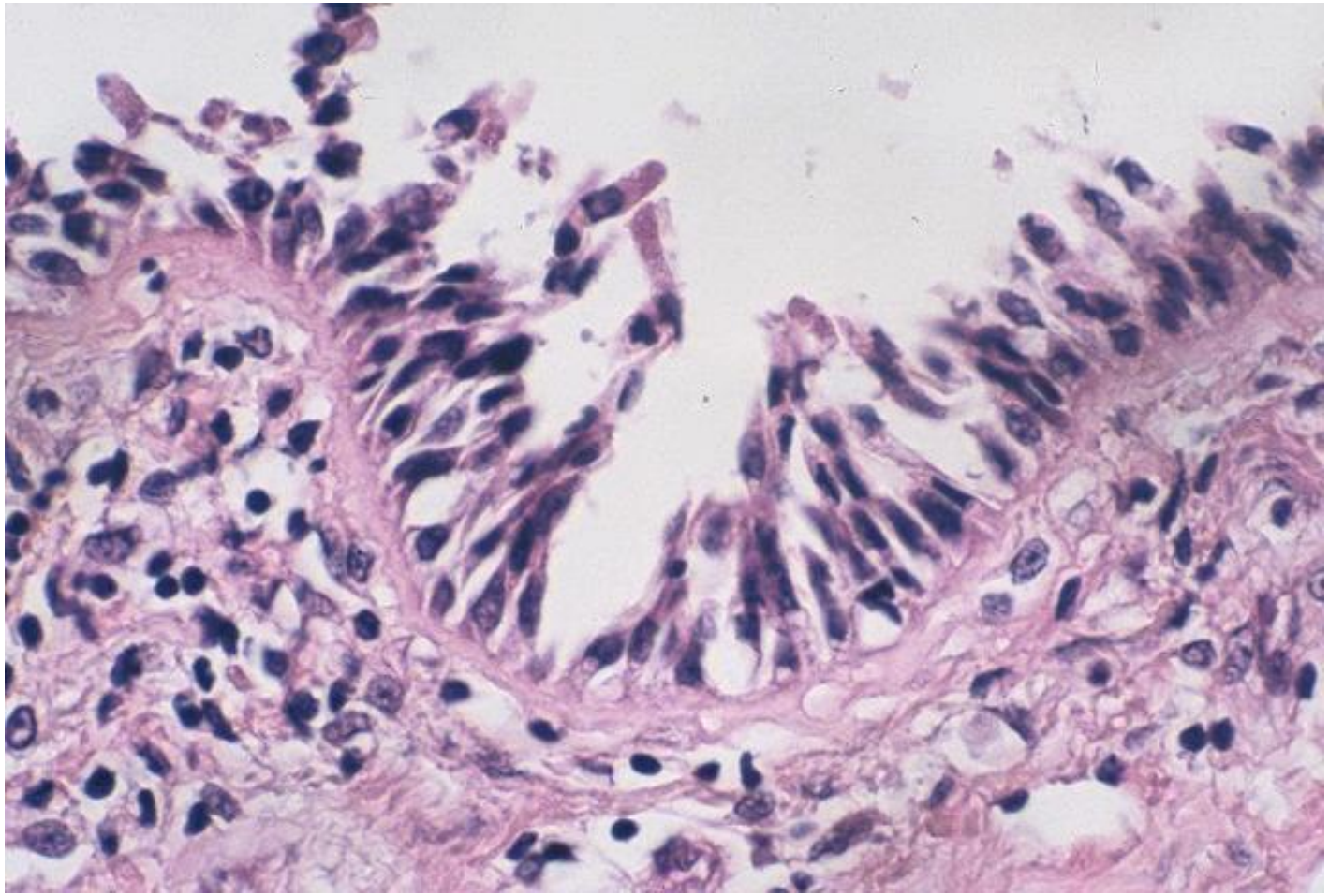


Fixation Incomplete



Time of fixation

Saline



Best Practice

Fixation Overview

- 10% Neutral Buffered Formalin NBF
  - Buffer pH 7.2-7.4
- Penetration
  - Formalin penetrates fast, but continues to cross link proteins for a long time after penetration is complete
  - **At least 6 hours, not more than 24-72 hours**
    - **includes time in processor**
    - **Document time**
- Volume 15-20:1 in a container.
- Temperature 22°C – 37°C

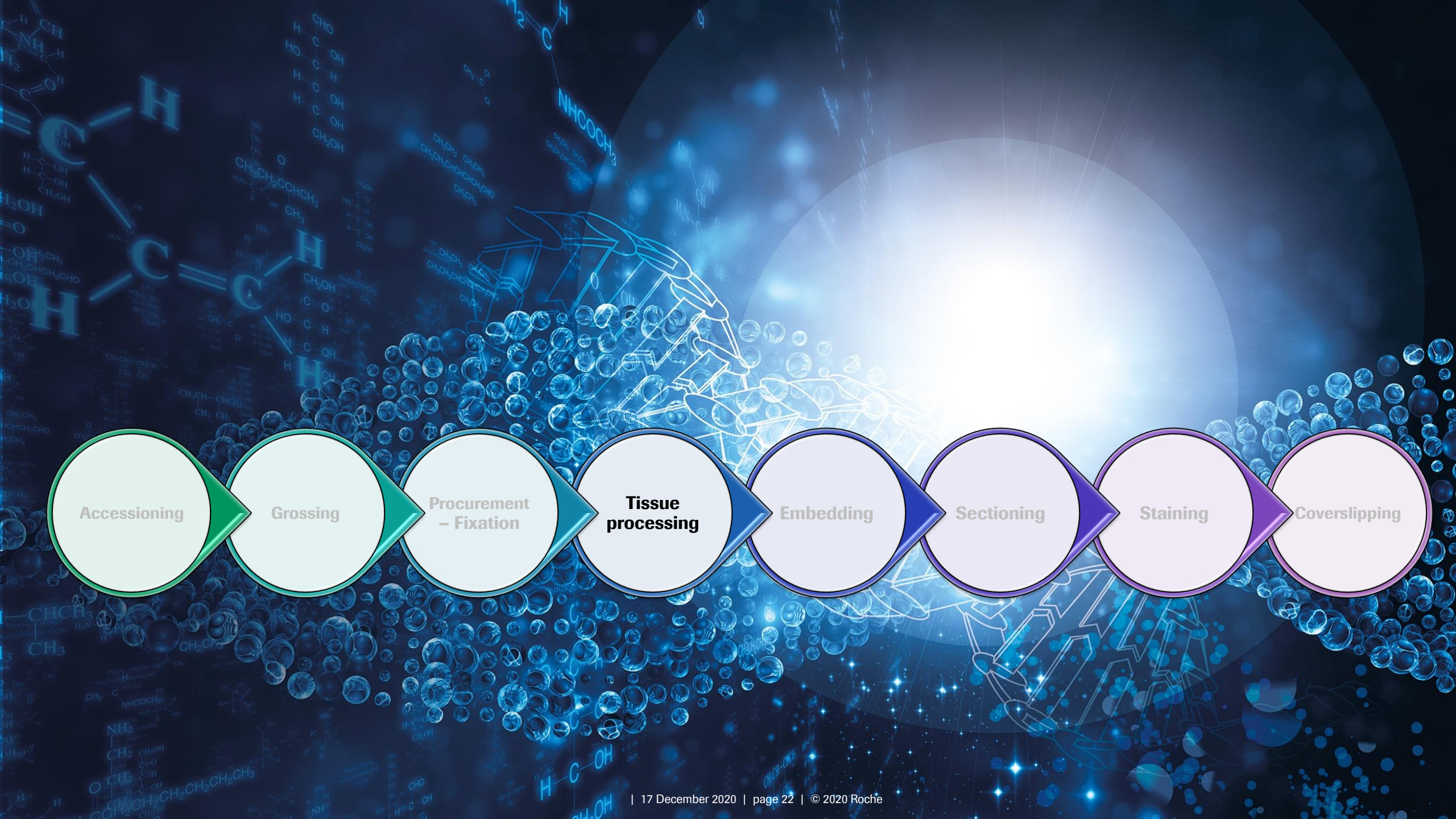
Other fixatives

Zinc Formalin – Z Fix

Alcoholic Formalin, AFA

Glyoxal based

Not recommended for IHC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Tissue Processing

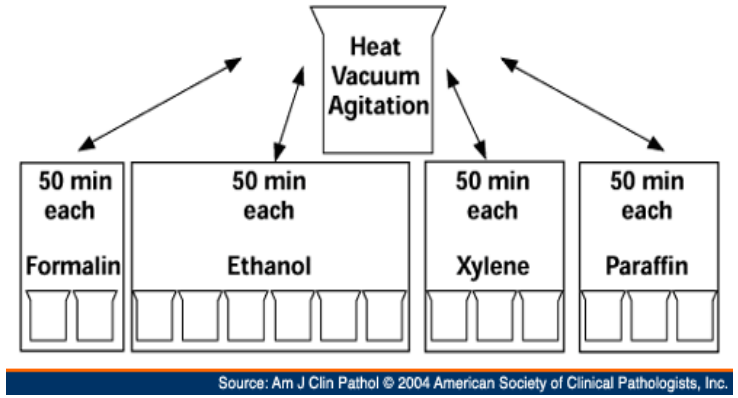
Processing Steps

1. Fixation: The purpose is to preserve tissues permanently in a state similar that it was taken form the body

2. Dehydration : Tissue samples are placed in a series of graded alcohols, usually beginning with 70% and ending with 100%.

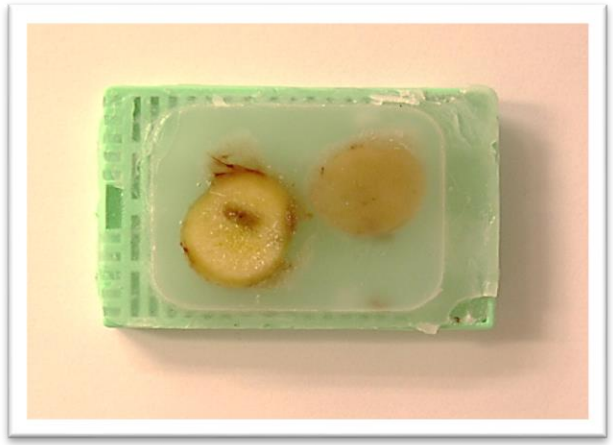
3. Clearing : An organic solvent (e.g.. Xylene) is used as an intermediary step because alcohol and paraffin are not compatible.

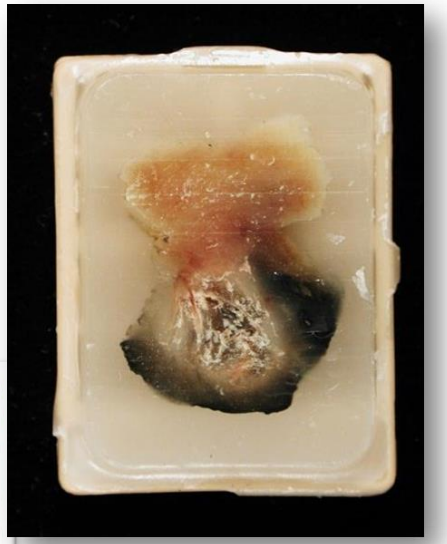
4. Infiltration : Tissue samples are then placed into changes of melted paraffin wax.

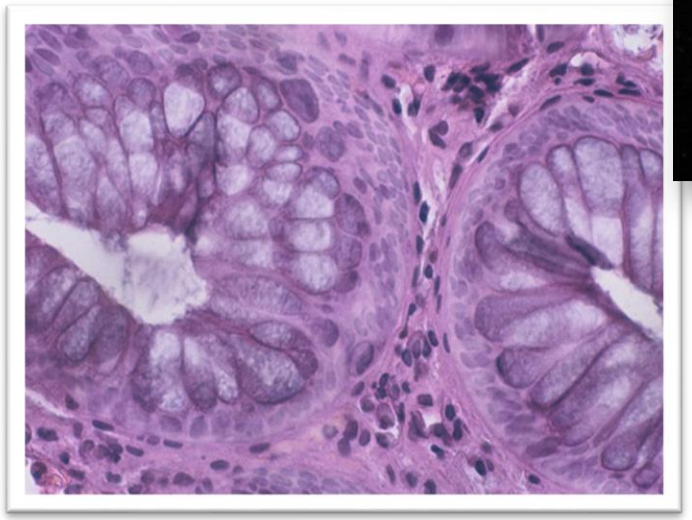


Tissue Processing

- Maintain reagent quality
- Use an appropriate schedule for tissue type
- Document rotation or reagent change
- Avoid over processing : Temperature/Vacuum/Pressure
- Not to exceed 37ºC
- Paraffin not to exceed 60ºC – not held for an extended time
- Kind of Wax

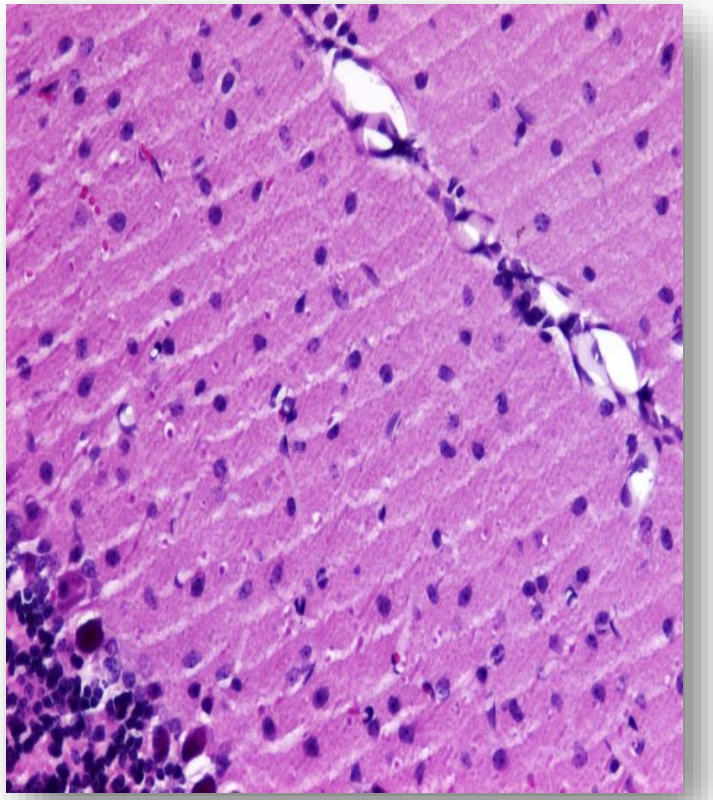






Best Practices Process

Common challenges with Grossing and Processing:
Over processed, dry and brittle tissue artifact



Description/Causes

- High temperature in processor, excessive time in the graded alcohols.
- High concentrations of Eosin on the processor may cause biopsy tissue to be brittle which requires excessive rehydration at microtomy, affects tissue section quality, ultimately affects staining.

Recommended Solutions

- Ensure tissue is not exposed to high temperatures.
- Biopsies should be processed separately to reduce time in alcohols and xylene.

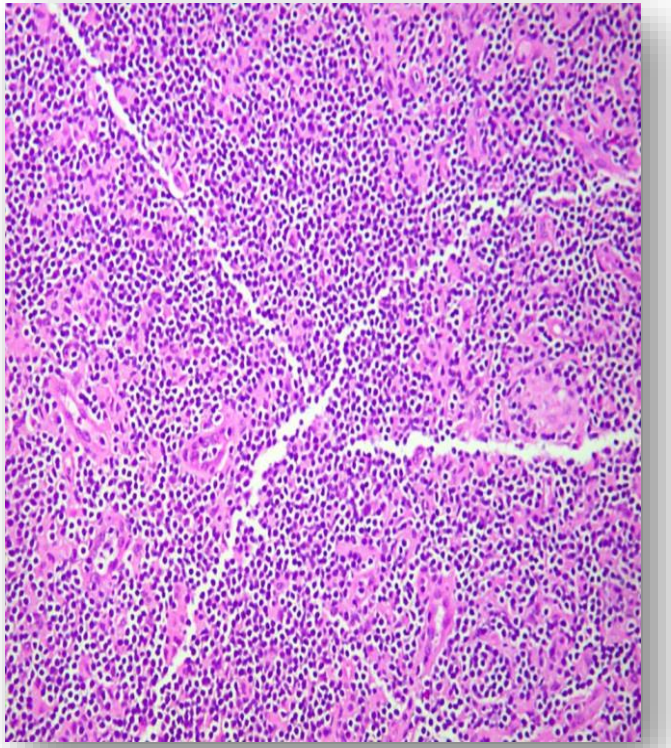
- Use heat only for paraffin, 2-4 °C above paraffin melting point.
- The temperature of the paraffin should be recorded daily.
- Ensure a specific protocol is defined based on biopsy, surgical, or fatty tissue. These should be processed separately from one another.

Highlights

- Avoid heat except for paraffin.
- Separate biopsies.

Best Practices Process

Common challenges with Grossing and Processing: Clearing and Infiltration



Description/Causes

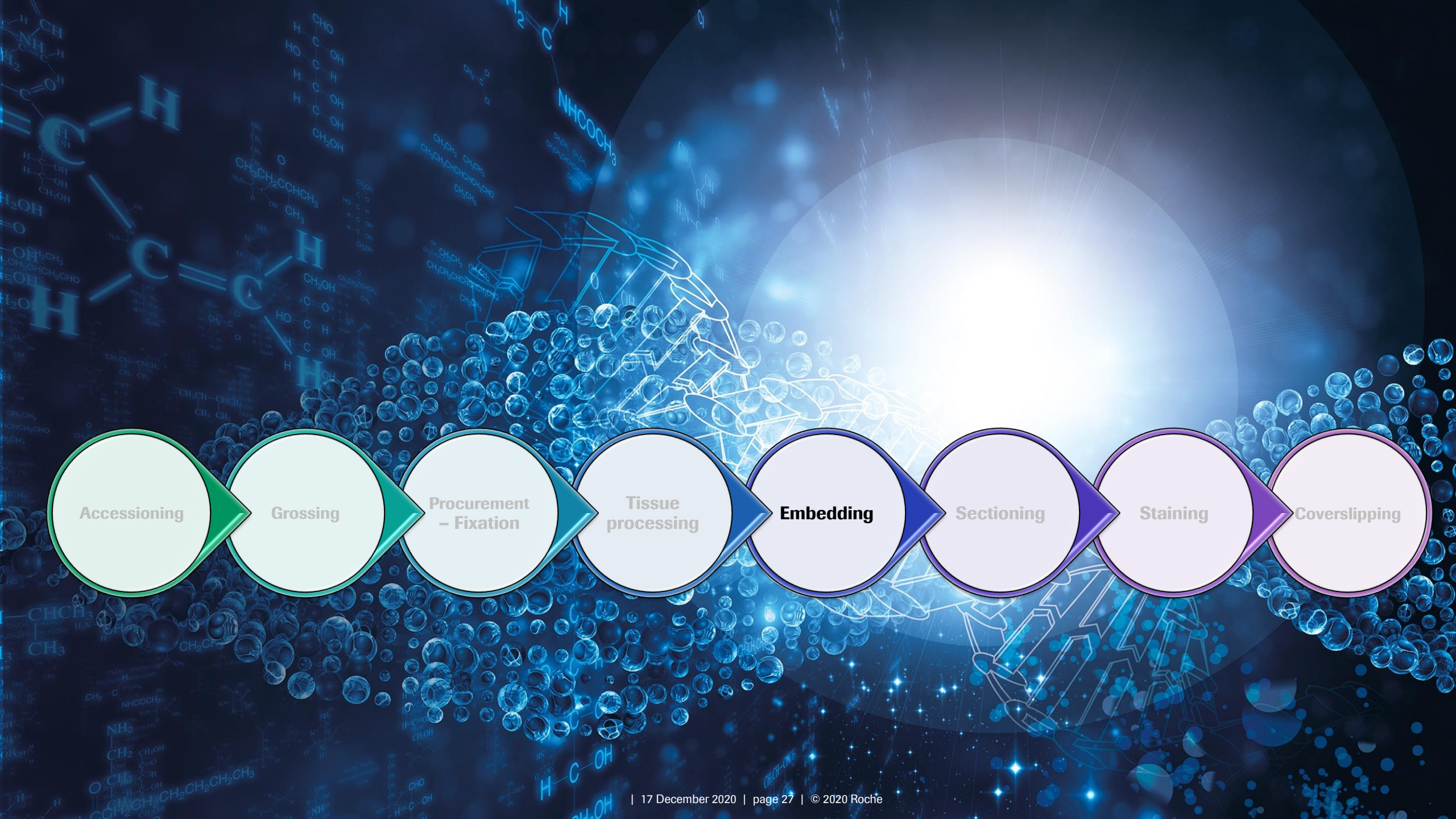
- Prolonged immersion in clearants (i.e.. xylene) may overharden specimen(s) and can cause artifacts
- Overheated paraffins cause tissue shrinkage and hardening

Recommended Solutions

- Duration in each station is dependent on type and size of specimen and efficiency of the processor.
- 2-3 changes of clearant for 15-60 minutes per change
- To prevent inactivation of tissue antigens, use paraffins with melting points below 58°C.
- Two to four changes of paraffin are required, ranging from 15-90 minutes each, depending on type and size of specimens. Infiltration time should be minimized.
- Poor quality paraffins interfere with the recovery of biomolecules.
- **Xylene-free processing considerations:**
  - Xylene-free protocols for tissue processing are dependent upon the evaporation of isopropyl (IPA) alcohol or acetone.
  - As in conventional processing, complete removal of water is equally important.

Highlights

- 2-3 changes of clearant for 15-60 minutes per change
- Use paraffins with melting points below 58C°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Embedding

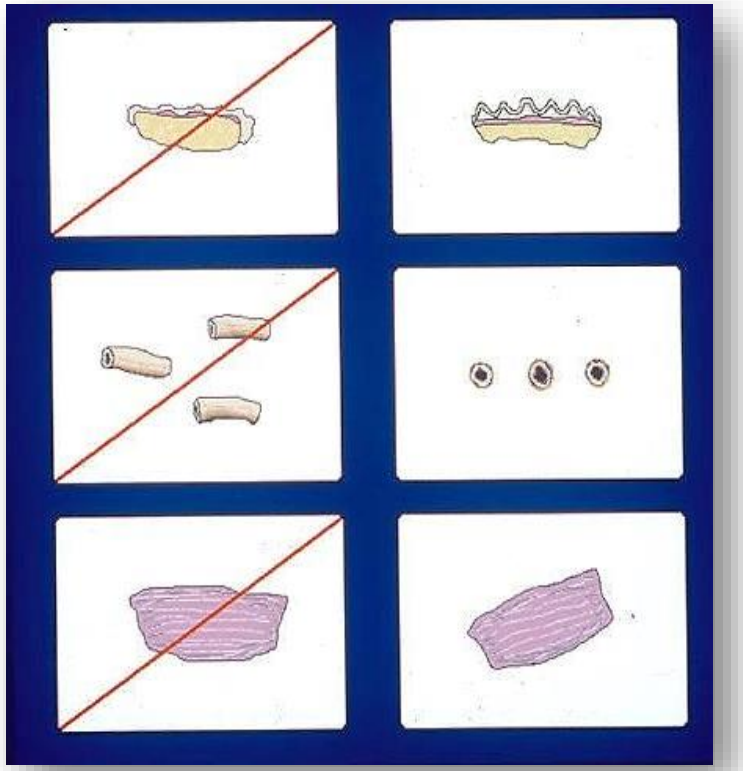
Tissue samples come off the tissue processor and are manually oriented in embedding molds.

The bottom of the cassette which contains the accession number is placed then over the mold.

- Avoid contamination- Open and embed one cassette at a time
- Clean forceps between each use
- Avoid excessive heat – melting point
- Check & document temperatures regularly
- Orient specimens correctly

Best Practices Process

Common challenges with Embedding: Poor orientation



Description/Causes

- Multiple pieces are not embedded in the same plane. Tissue pieces in different planes creates a lack of uniformity and results in an incomplete section. All pieces are not flat & level.

Recommended Solutions

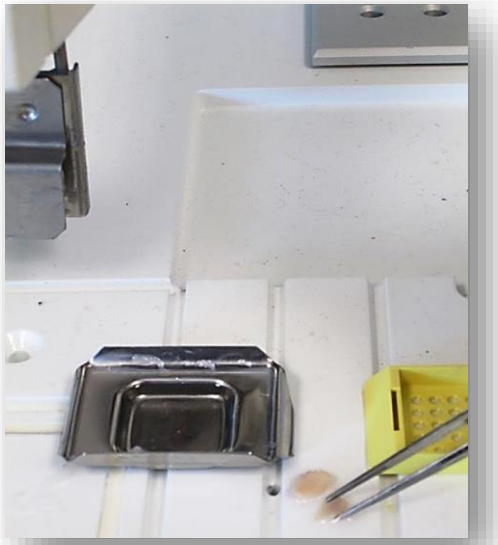
- Tissue should be embedded in the center of the mold with a margin of paraffin around the tissue that is relative to the size of the tissue (suitable but not excessive).
- If not otherwise instructed, place the tissue with the side that is down in the processing cassette down in the mold.
- Tissues with a wall, such as cysts, gall-bladder, and gastrointestinal tract, must be embedded on edge so that all layers are visible.
- Tubular structures are embedded on end so that the cross-section of the lumen displays all layers.
- Skin should be oriented so that the epithelium is facing one side of the mold. If more than one piece of skin is to be embedded in the same mold, then the epidermis of all pieces should face the same side.
- While embedding, it is important that light pressure be applied over the entire specimen during the orientation and initial chilling so that the tissue will be flat. Tamping the tissue is necessary to get an even surface.
- All tissue pieces should be flat, level, and on the same plane.
- Angle tissue to minimize surface area of the blade.

Highlights

- Embed in center of mold.
- Placement should enable visualization of desired tissue structure.
- Place flat on same plane in mold.

Best Practices Process

Common challenges with Embedding: Artifacts



Description/Causes

- Small friable tissues may suffer from heat induced artifact and distortion of cellular morphology.
- Placement of tissue on the hot plate surface also increases contamination risk.

Recommended Solutions

- Tissue should not be placed on the embedding center hot plate between opening the cassette and placing tissue in the mold.
- Orientation of tissue should occur in a clean mold, using clean forceps.

Highlights

- Specimens may be lost or may suffer heat artifact.

Description/Causes

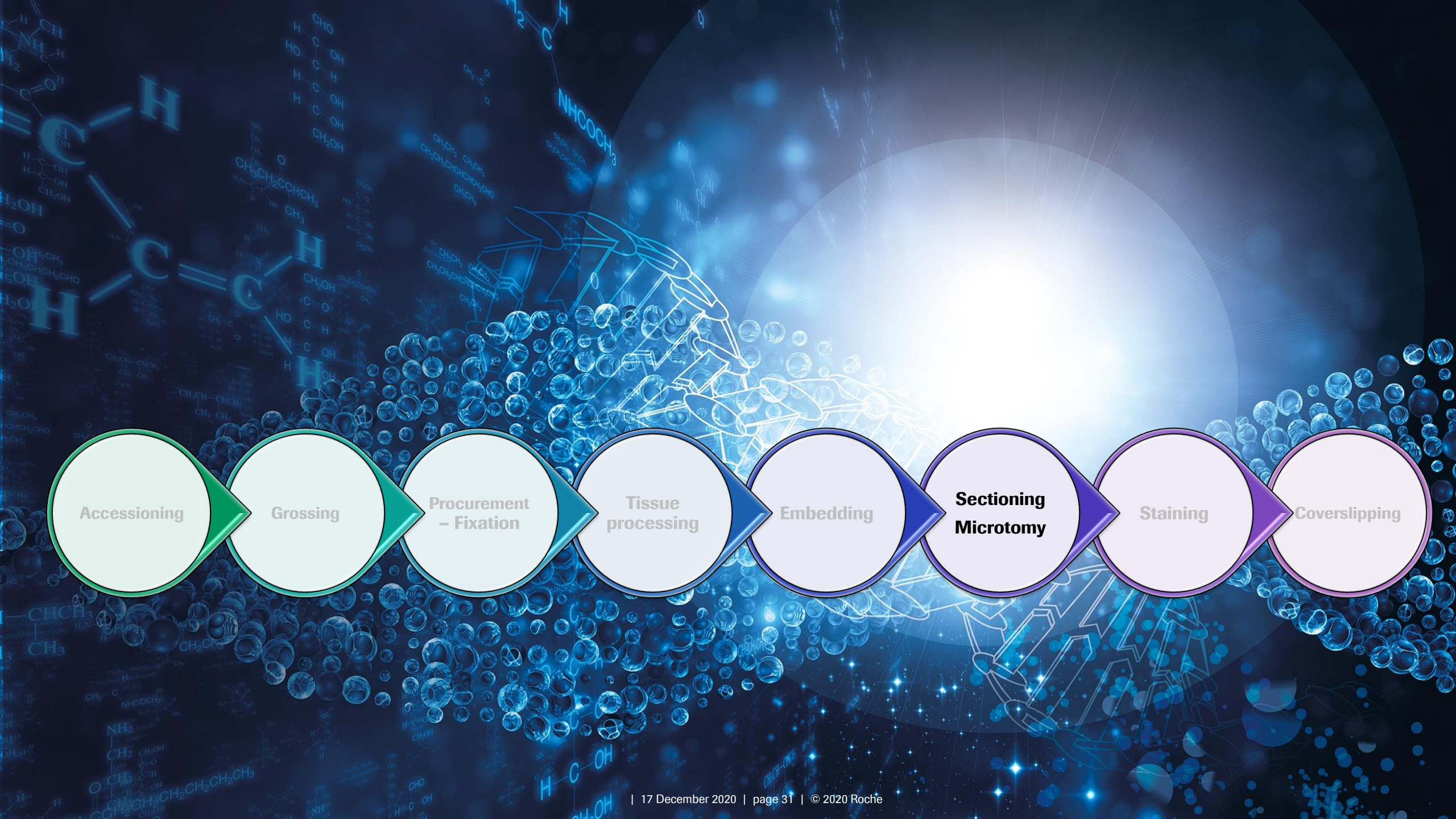
- Processed blocks are transferred and held in the heating chamber of the embedding center. The chamber is dry, containing no melted paraffin.

Recommended Solutions

- Specimens should be immersed and held in paraffin prior to the embedding step.
- The temperature of the paraffin should be recorded daily.

Highlights

- No tissue directly on hot plate.
- Transfer tissue directly into the mold.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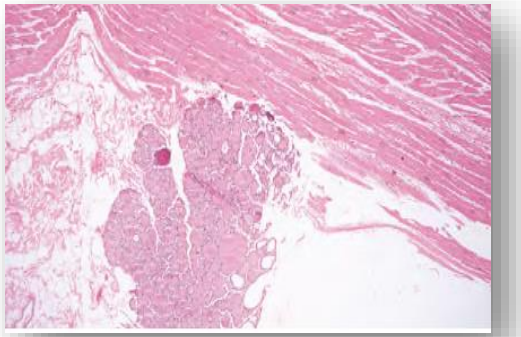
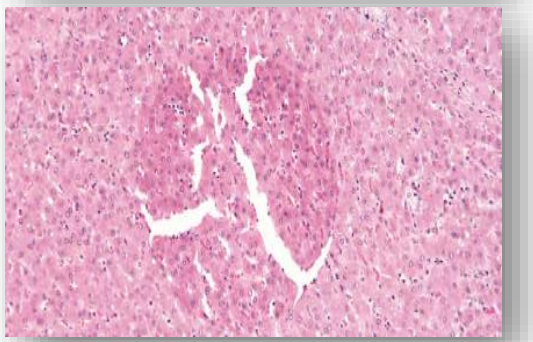
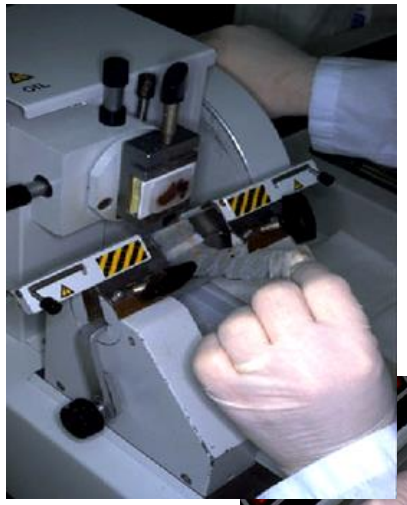
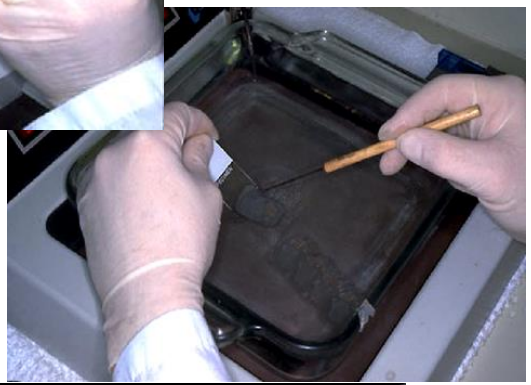
Microtomy

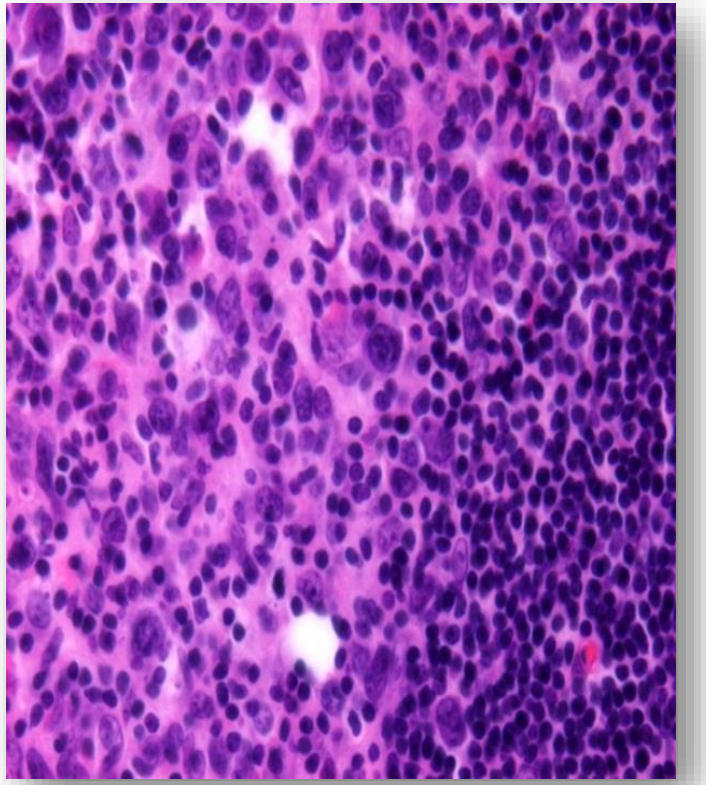
- A microtome is used to cut very thin precise paraffin sections. (3-6 microns)
- Due to friction, heat is generated on the knife to form a wax ribbon of tissue sections.




Best Practices Process

Common challenges with Microtomy: Section cut too thick or thin



Description/Causes

- Sections cut too thin may yield light staining.
- Sections cut too thick, having more than one cell layer, may appear over stained.
- Sections that are too thick (greater than 4µm) may result in incomplete deparaffinization.
- Thick sections have a tendency to fall off the slide due to inadequate drying.

Recommended Solutions

- Define a standard microtomy protocol. Laboratories should establish cutting guidelines for all specimen types:
  - Routine – 3-4µm,
  - IHC – 4µm,
  - Bone marrow – 3µm
  - Renal – 2µm
- Guidelines for the number of sections/ribbons per slide.
- Avoid using first and last section in the ribbon.
- Microtomes should be serviced according to manufacturer's recommendations.

Highlights

- Cut tissue 3-4 µm thick.
- Do not use first and last section in the ribbon.
- Laboratories should have standards for section thickness

Best Practices Process

Common challenges with Microtomy: Water trapped on slide/nuclear bubbling

Description/Causes

- Trapped water between tissue and slide can cause nuclear bubbling.
- Poor charge on the glass slide contributes to trapping water between the glass and the tissue section making it difficult to remove.

Recommended Solutions

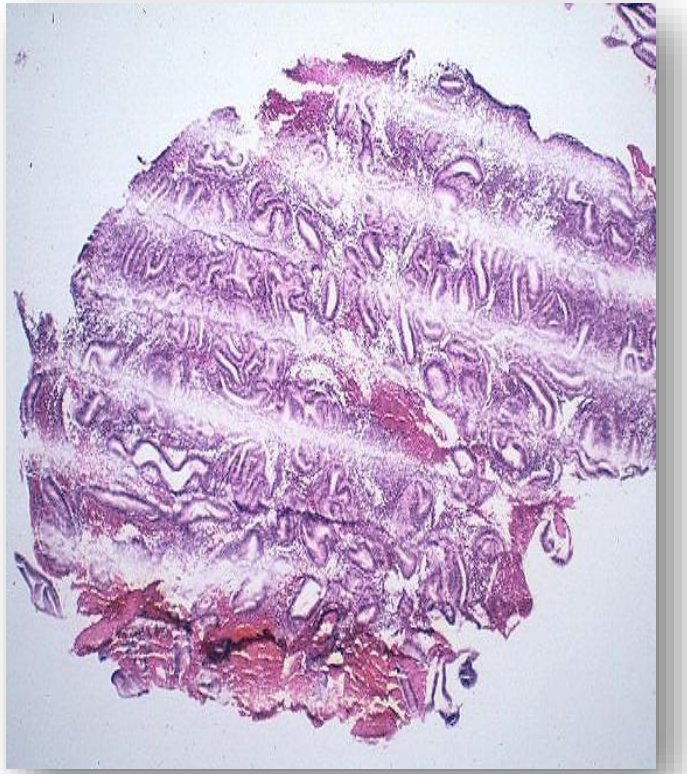
- Choosing a slide that is NOT hydrophobic- Allows proper water drainage and tissue drying prior to staining.
- Remove water trapped between tissue and slide.
- Wick slides – Slides should be allowed to drain before being dried for staining. Removal of water will assist with tissue adhesion during the horizontal/vertical stain process.

Highlights

- No hydrophobic slides.
- Proper drainage
- Check slide for trapped water.

Best Practices Process

Common challenges with Microtomy: Venetian blind effect of tissue



Description/Causes

- Can be caused by hard or over-processed tissue, a dull blade, or too much clearance angle.
- Block is loose in the chuck or the blade is not secure in the holder. Poorly maintained microtomes increase likelihood of stripped or worn parts causing inconsistent advancement and thickness.

Recommended Solutions

- Remedies include improving tissue processing (see grossing section), replacing blade, or altering angle of blade.
- Ensure microtome blade is consistently sharp by replacing frequently.
- Microtomes should be serviced, follow manufacturer's guidelines.
- Ensure block and blade are securely fastened.
- Ensure clearance angle is in line with microtome's manufacturer's guidelines.
- Practice good microtomy - steady motion while turning the microtomy wheel and visual evaluation of ribbon quality while cutting.
- In the presence of heavily calcified tissue, decal solution can be used (but not one that contains hydrochloric acid). Rinse block prior to cutting to remove decal solution (this is not a good practice as it interferes with immuno staining).

Highlights

- Fresh, sharp blades
- Proper blade angle
- Consistent microtomy wheel motion
- Secure blade, minimize vibration

Best Practices Process

Common challenges with Microtomy: Poor placement of tissue

Description/Causes

- Poor placement of tissue can result in inconsistent staining along slide edges and coverslipping coverage issues.

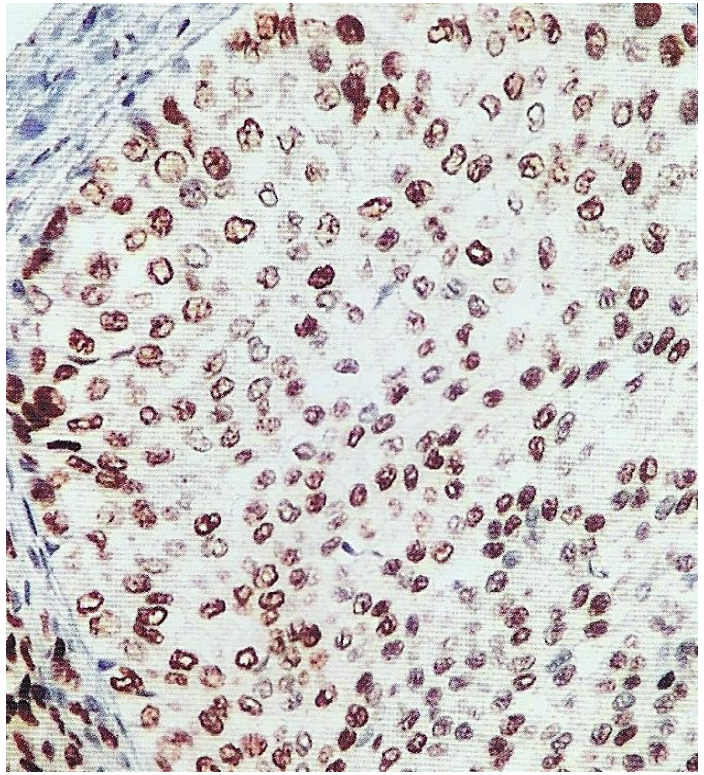
Recommended Solution

- Place tissue on center of slide.

Highlights

- Center tissue on slide.

Slide Stability



If “pre-cut control slides” are not dried or stored at appropriate temperature, the result will be continued loss of antigenicity as storage continues.

Formal and Literature-Based Recommendations

for Specimen Fixation and Processing Variables for Optimal Staining

Storage

Pre-analytic variable	Published Guidelines and Recommendations **ASCO/CAP; CLSI**	Literature-Based Recommendations
• Storage of cut sections	7 days or < 6 weeks	< 6 days
• Storage of paraffin blocks	Indefinitely	< 25 years

Days	20˚C
Week	4˚C
Month	-20˚C
Year	-80˚C

Engel KB, Moore HM. *Arch Pathol Lab Med*. 2011;135:537-543

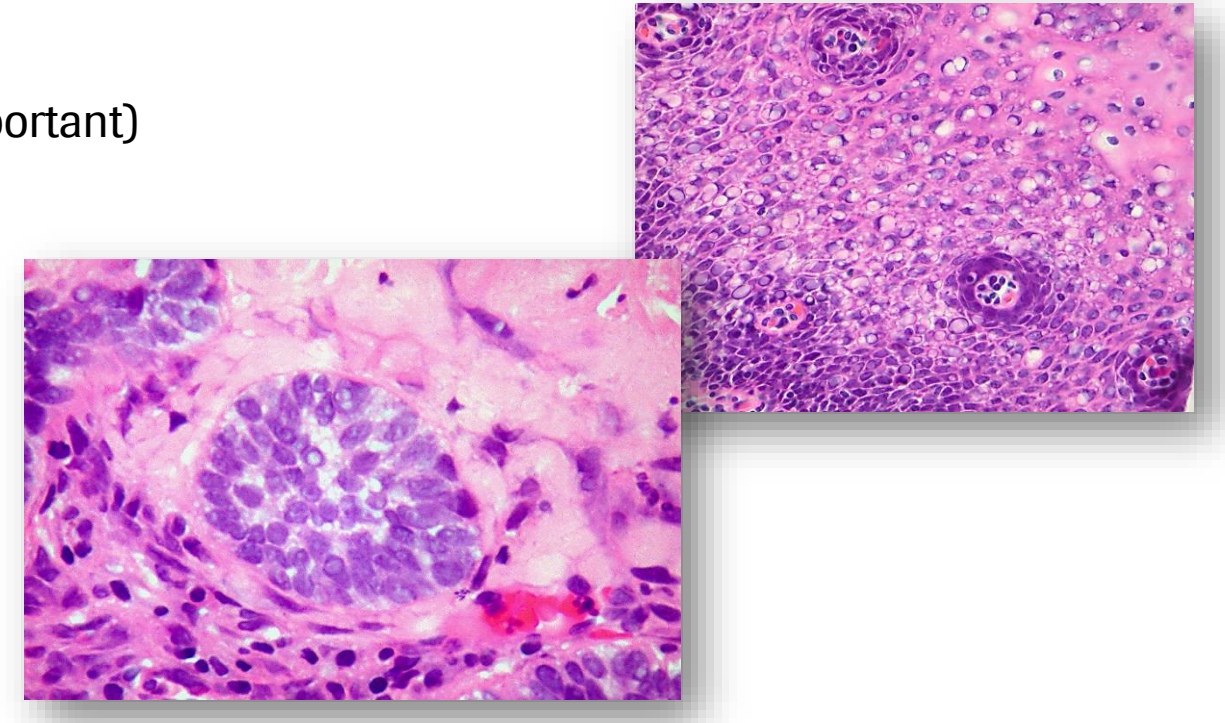
Sectioning – Slide Drying

The slides are then placed in a oven to evaporate the water on the slide and to properly adhere the section on the slide.

Recommendation:

60˚C for a maximum of 60 minutes, 37˚C for a maximum of 24 hours, or at ambient temperature for 24 hours or longer

- Drain before drying (very important)
- Monitor drying temperature



Best Practices Process

Common challenges with Staining: Shortened tissue drying time impedes tissue adherence

Description/Causes

- Inadequate drying time can affect tissue adherence.
- Staining quality can be negatively affected by improper drying processes

Recommended Solution

- It is recommended that freshly cut sections be air-dried and water wicked from the slide for 10-15 mins before placing into a drying oven.
- Water between the section and slide must be removed to avoid artifacts.
- The temperature of the drying oven should be maintained just above the melting point of the paraffin.
- Published guidelines recommend drying slides for 24 hrs. at room temperature or in an oven between 50°C-60°C for 30-60 min.

Highlights

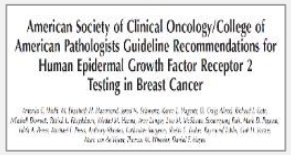
- Standardize drying time.
- Dry slides for 24 hrs. at RT or in an oven between 50°C-60°C for 30-60 min.

Pre-analytic guidance and resources

CAP

Breast cancer guidelines

2015


American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer

Updated 2018


Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer

American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update

[Source](#)

IASLC

Lung cancer EGFR and PD-L1 guidelines

2017

[Source 1](#); [Source 2](#)

CAP NSH

Surgical pathology guide

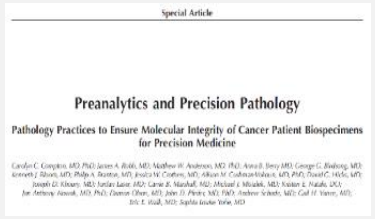

Practical Guide to Specimen Handling in Surgical Pathology

2020

[Source](#)

CAP Special Article

Pre analytics and precision pathology


Special Article

Preanalytics and Precision Pathology

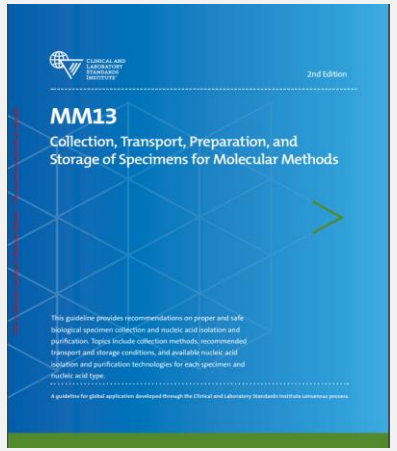
Pathology Practices to Ensure Molecular Integrity of Cancer Patient Biospecimens for Precision Medicine

2019

[Source](#)

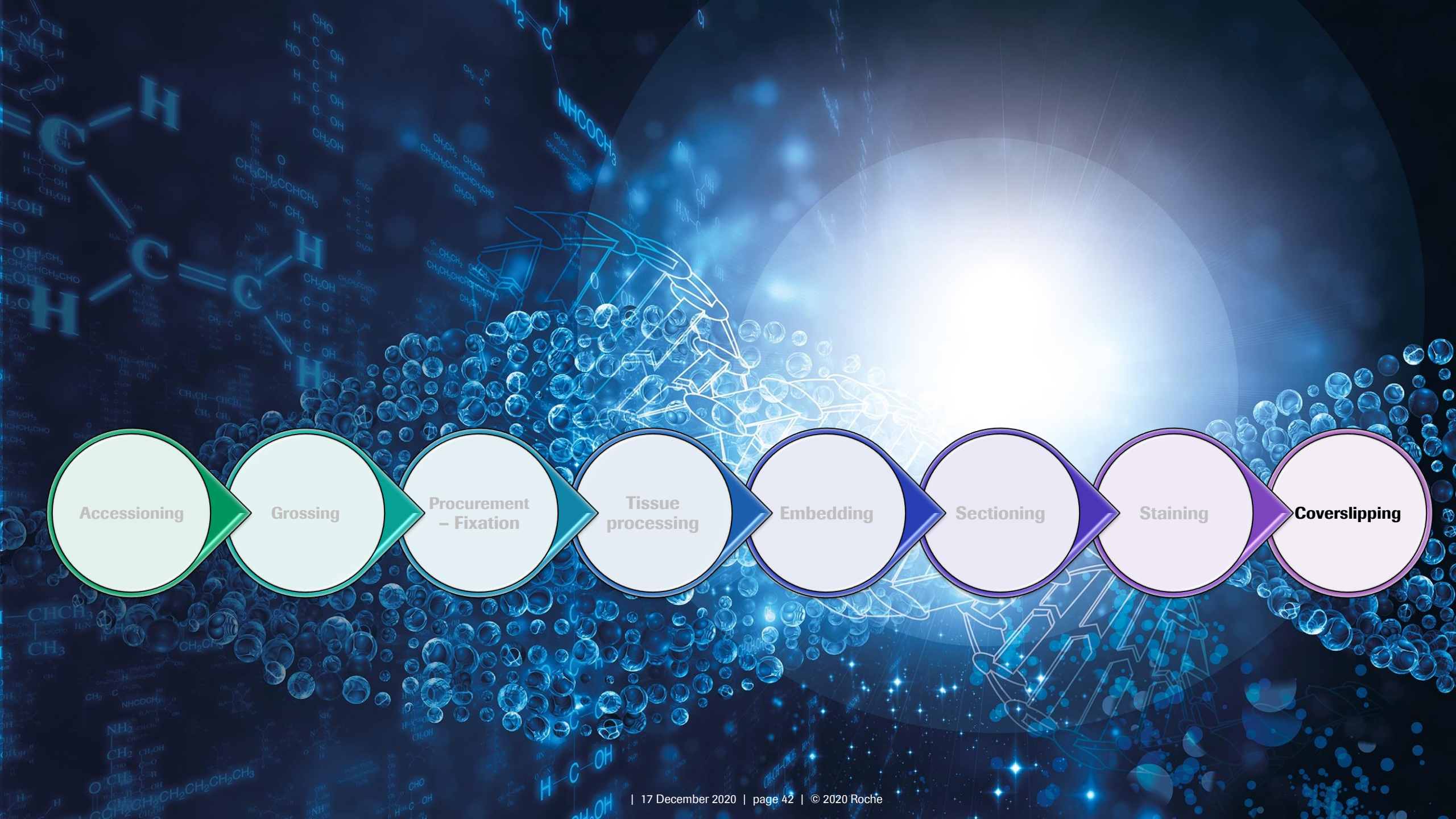
CLSI

Molecular Standards



2020

[Source](#)


Accessioning
Grossing
Procurement – Fixation
Tissue processing
Embedding
Sectioning
Staining
Coverslipping

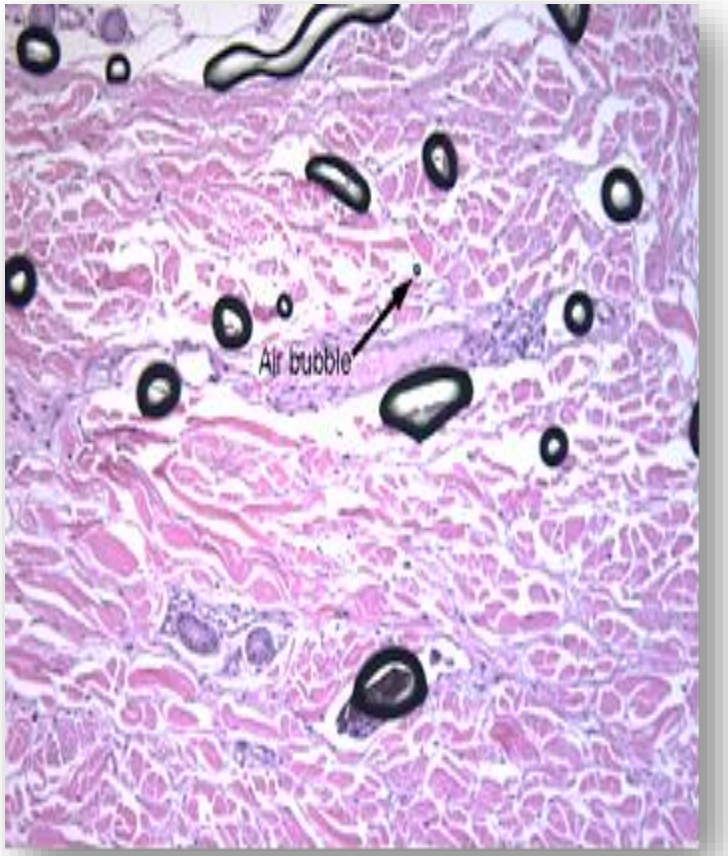
Coverslipping

The stained section on the slide is covered with a thin glass coverslip that will protect the tissue section and allow the slide to be archived.

Dehydration and mounting media may have an impact on stain fade.

Best Practices Process

Common challenges with Coverslipping: Bubbles



Description/Causes

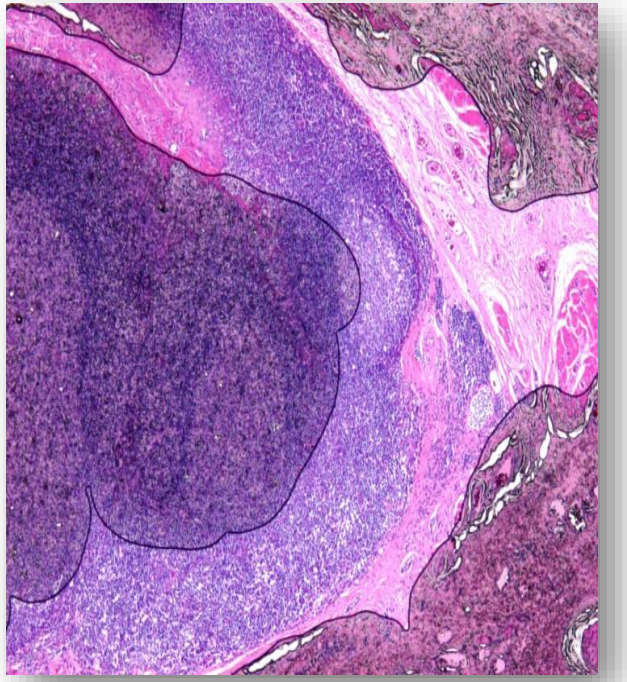
- Bubbles occur when air is trapped between the stained section and the coverslip. Causes include: mounting media too thick, poor technique, poorly placed mounting media, poorly placed coverslip.

Recommended Solution

- Review technique of coverslipping
- Utilize proper mounting media for coverslipping

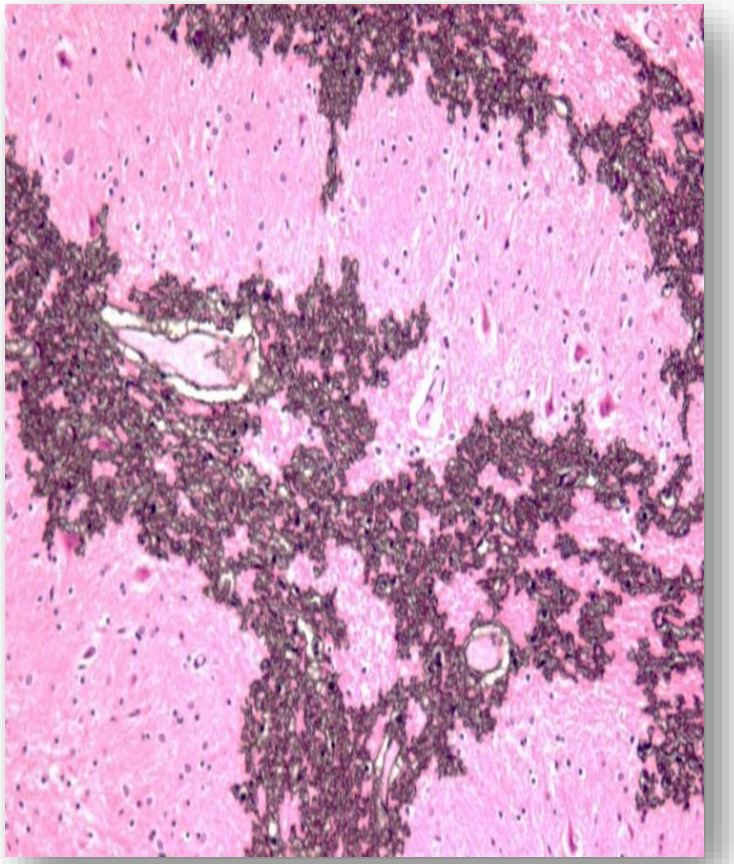
Highlights

- Utilize fresh mounting media.
- Do not dilute mounting media with xylene.



Best Practices Process

Common challenges with Coverslipping: Cornflaking effect



Description/Causes

- Corn flaking effect occurs when tissue dries before mounting media is added.

Recommended Solution

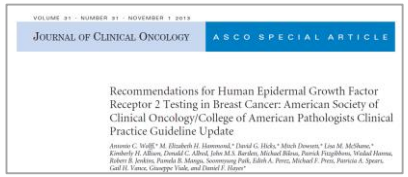
- Keep slides immersed in xylene prior to coverslipping

Resources

ASCO/CAP

Breast cancer guidelines

2013


JOURNAL OF CLINICAL ONCOLOGY ASCO SPECIAL ARTICLE

Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update

Updated 2018


Special Article

Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer

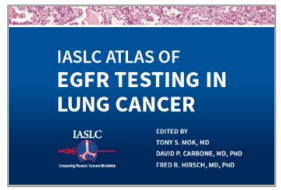
American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update

| Wolff A et al. J Clin Oncol 2013

| Wolff A et al. Arch Pathol Lab Med 2018

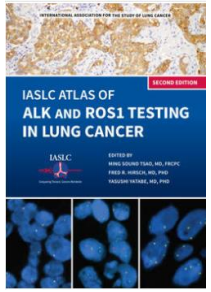
IASLC

Lung cancer testing guidelines

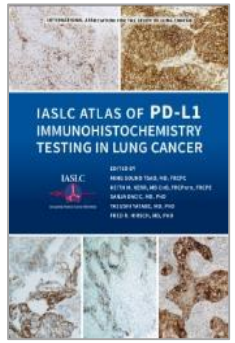

IASLC ATLAS OF EGFR TESTING IN LUNG CANCER

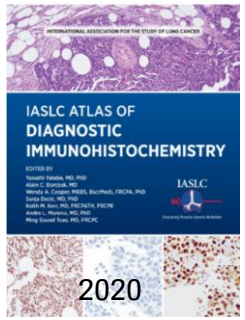
IASLC

EDITED BY TONY S. MOK, MD DAVID P. CARBONE, MD, PhD FRED R. HIRSCH, MD, PhD


IASLC ATLAS OF ALK AND ROS1 TESTING IN LUNG CANCER

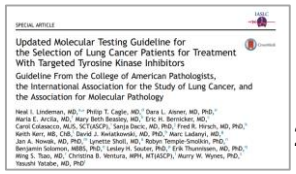
SECOND EDITION


IASLC ATLAS OF PD-L1 IMMUNOHISTOCHEMISTRY TESTING IN LUNG CANCER


IASLC ATLAS OF DIAGNOSTIC IMMUNOHISTOCHEMISTRY

IASLC

2020


SPECIAL ARTICLE

Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment With Targeted Tyrosine Kinase Inhibitors

Guideline From the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology

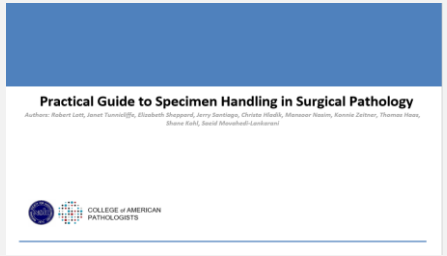
2017

| Lindeman N et al. J Thorac Oncol 2017

| https://www.iaslc.org/research-education/publications-resources-guidelines/

CAP/NSH

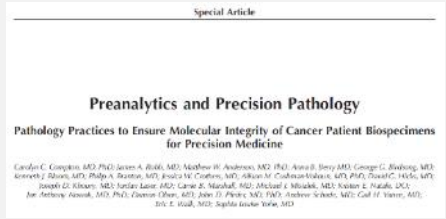
Guide to Specimen Handling in Surgical Pathology


Practical Guide to Specimen Handling in Surgical Pathology

COLLEGE of AMERICAN PATHOLOGISTS

2020

Pre-analytics and precision pathology


Special Article

Preanalytics and Precision Pathology

Pathology Practices to Ensure Molecular Integrity of Cancer Patient Biospecimens for Precision Medicine

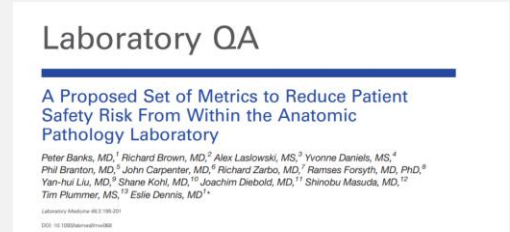
2019

| Lott R https://cap.objects.frb.io/documents/practical-guide-specimen-handling.pdf

| Compton C et al. Arch Pathol Lab Med 2019

Publications

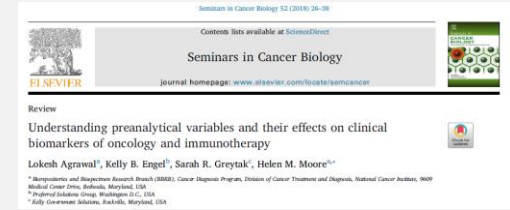
Patient safety metrics


Laboratory QA

A Proposed Set of Metrics to Reduce Patient Safety Risk From Within the Anatomic Pathology Laboratory

2017

Pre-analytic variables effects on oncology biomarkers


Seminars in Cancer Biology

ELSEVIER

Review

Understanding preanalytical variables and their effects on clinical biomarkers of oncology and immunotherapy

Lokesh Agrawal, Kelly B. Engel, Sarah R. Greytak, Helen M. Moore

2018

| Banks P el al. Lab Med 2017

| Agrawal L et al. Semin Cancer Biol 2018

Q&A

Part 2 : *Effects of Poor Tissue Handling on Testing*

Thank you

beth.sheppard@roche.com

