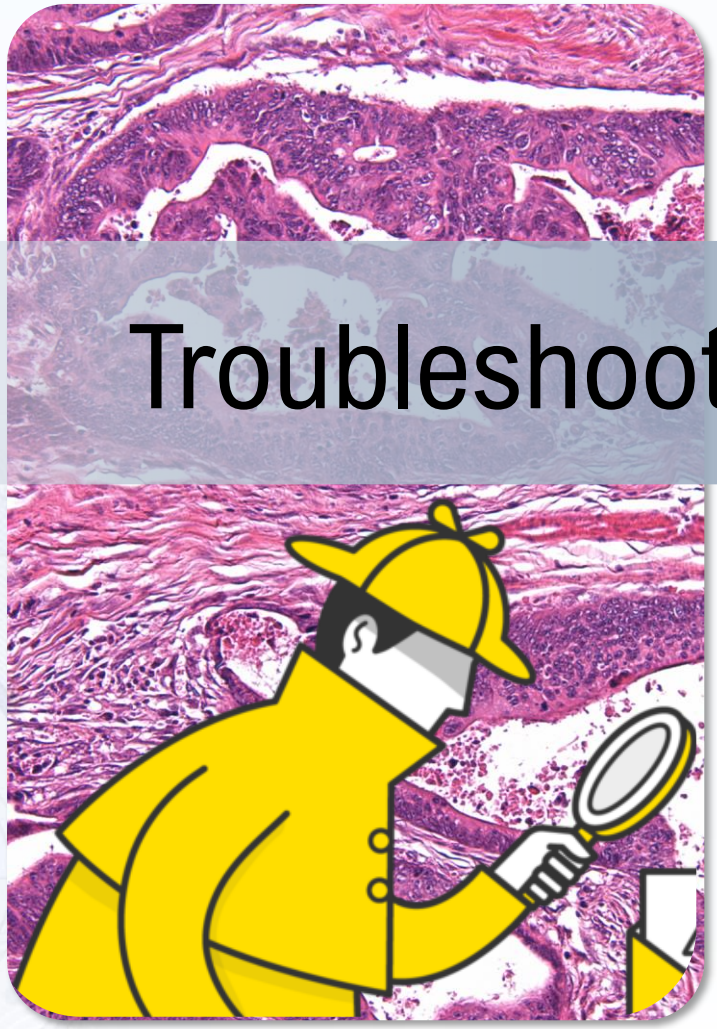


Troubleshooting Routine Histology

A Guide on How to Avoid Common Mistakes

Andrew R. Lisowski MS, HTL (ASCP)

LEARNING OBJECTIVES

- Why troubleshooting might be challenging
- Complexity of a typical histology workflow
- Proper techniques and common mistakes in:
  - Grossing
  - Fixation & decalcification
  - Processing
  - Frozen and paraffin embedding
  - Frozen and paraffin microtomy
  - Staining
  - Coverslipping
  - Storing & archiving
- Summary

THE ART OF TROUBLESHOOTING

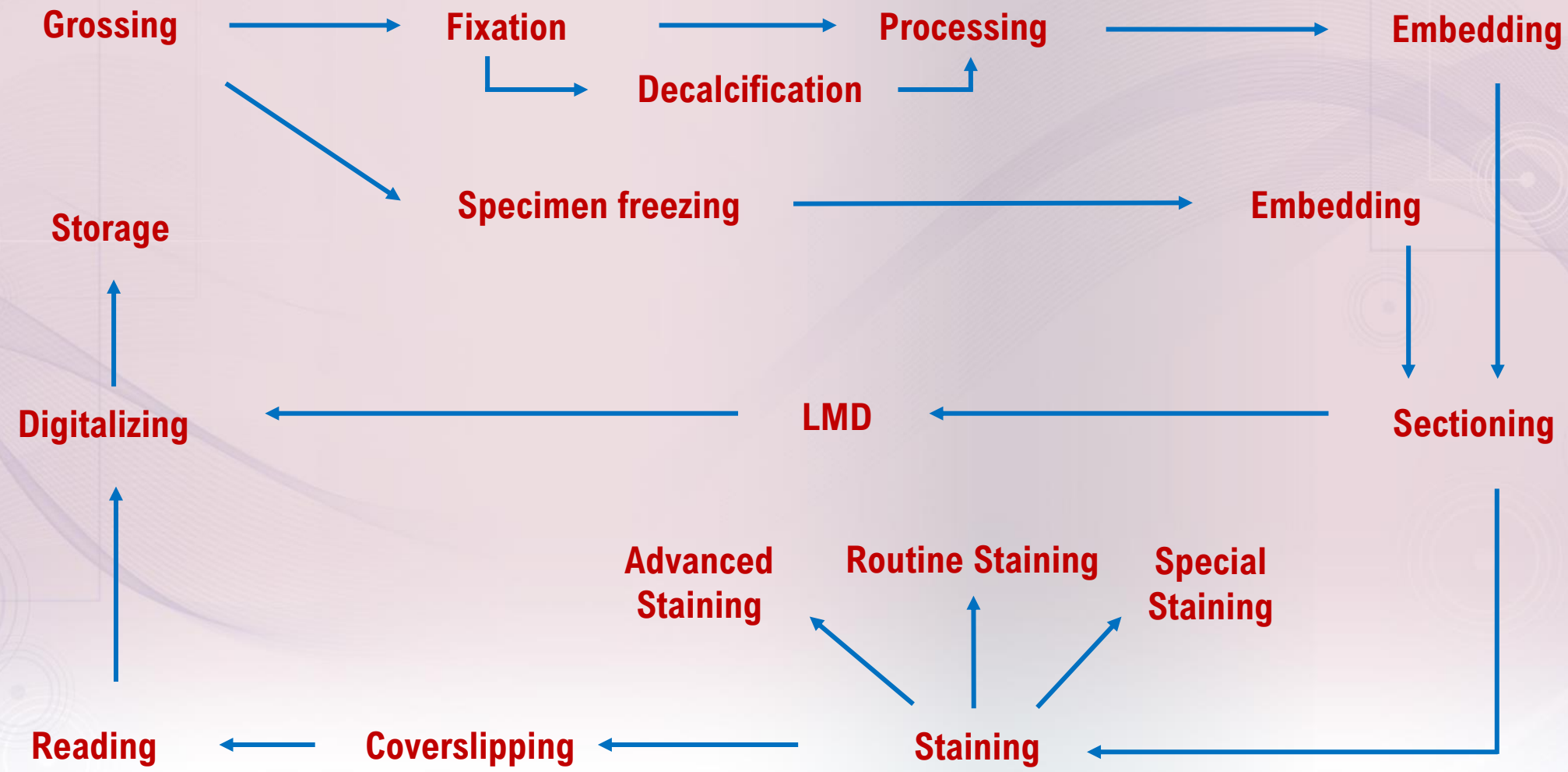
Since the histology workflow is very **complex** and involving many different consumables, it is often very challenging to correctly troubleshoot the exact problem.

Consumables that are dedicated to specific steps **might vary** in chemical makeup, physical properties, and even differ in size and color.

Adding a **variety of instruments** on the market with - their different capabilities, ease of operation, and customer interface - makes this troubleshooting even harder.

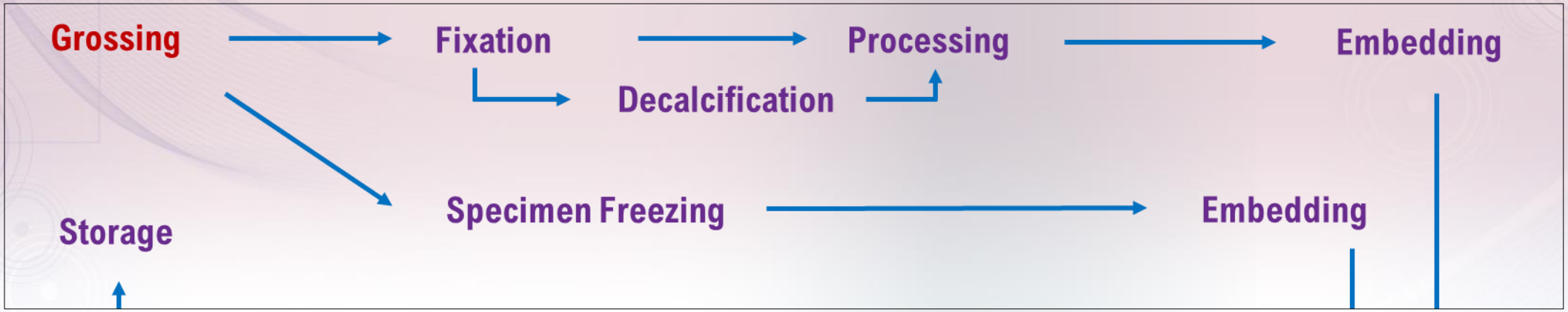
An **educated** operator will minimize repeated work, decrease time and laboratory costs, improving performance in patient care.

HISTOLOGY WORKFLOW STEPS



GROSSING

- When handling fresh tissue, handle it gently to avoid mechanical trauma.
- Never allow the specimen to dry out; moisten with saline if needed.
- Specimen that needs to be trimmed must “comfortably” fit a histology cassette.
- Oversized specimens need to be scored or opened to allow for optimal fixative penetration into thicker areas of the specimen.
- Use an appropriate size container with an adequate volume of fixative to tissue (ratio of at least 20:1).



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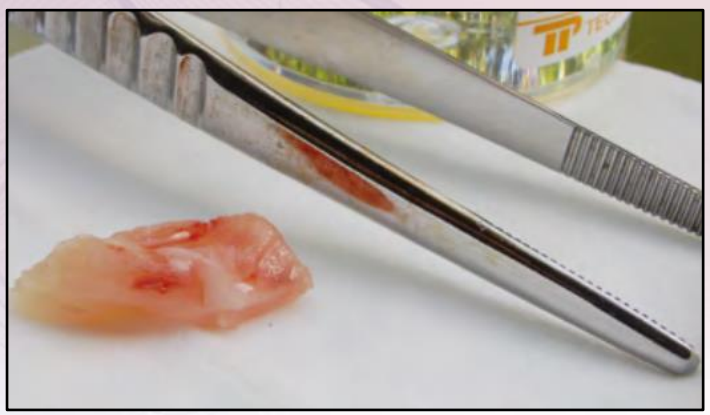
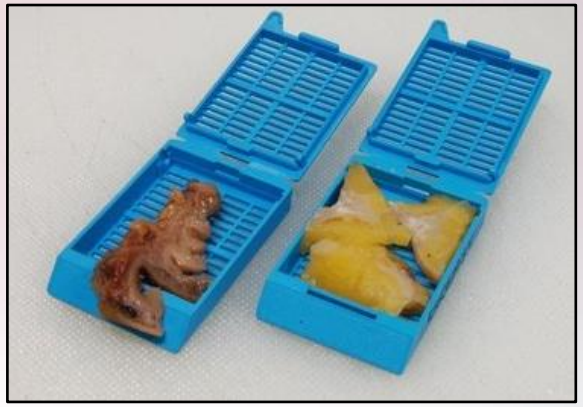
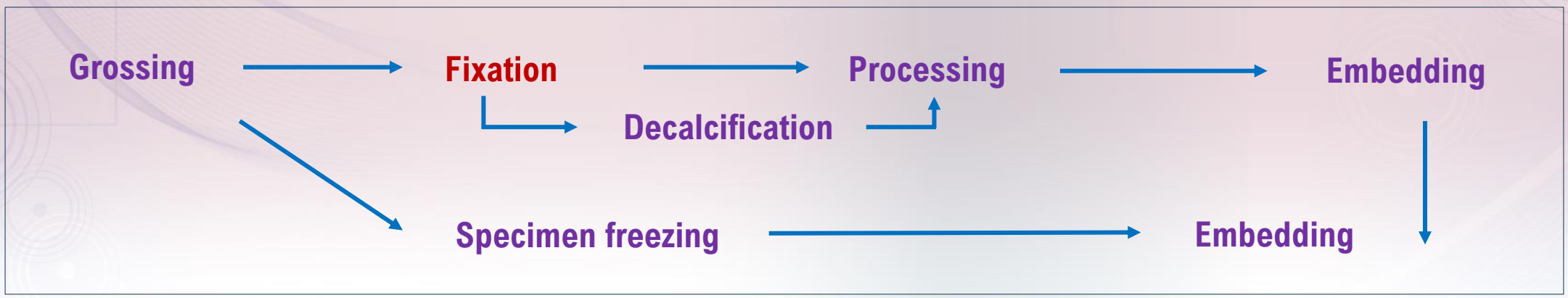



FIXATION

- Use correct fixative or fixative designed for tissue type.
- Use fixative of high quality and at the known pH. If formalin is used at acid pH it rapidly produces “formalin pigment” (acid formaldehyde hematin) by reaction with hemoglobin.
- When using non-coagulating or cross-linking fixative for samples that will be subjected to IHC procedure, carefully monitor fixation time and remember about adding an antigen retrieval step.
- Keep in mind proper fixative to tissue ratio (ideally, 20:1).
- Remember that fixation time is crucial and specific to tissue size and type.



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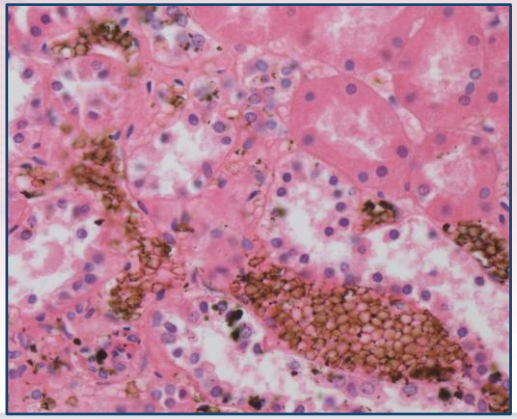
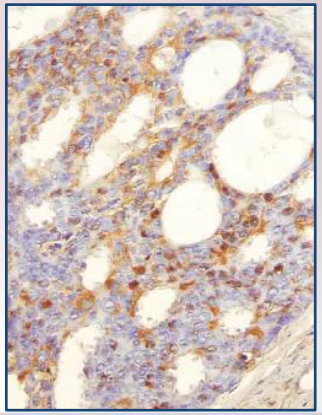
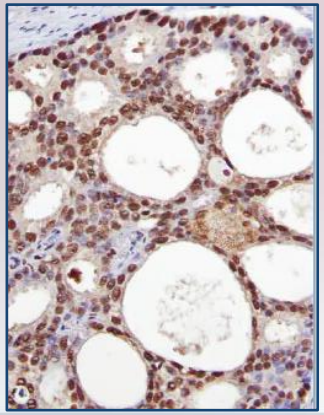
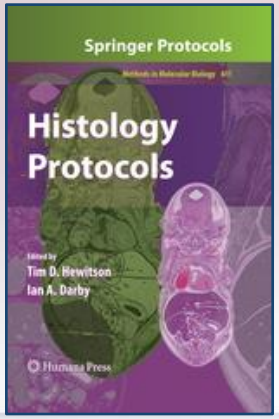
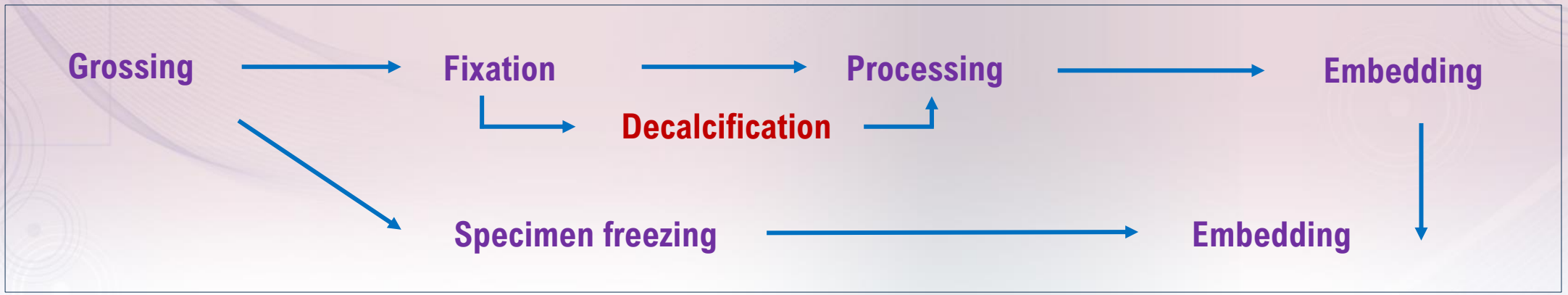





DECALCIFICATION

- Bone size determines the decalcification agent that must be used (strong vs. weak acid).
- Excessive duration allowed for decalcification will lead to loss of morphology.
- Inadequate time will make sectioning difficult, if not impossible.
- Samples for IHC or ISH assays might require special decalcification agent (EDTA).
- Some non-calcified tissues might need decalcification when calcium salts are unexpectedly present (e.g., kidney stones, calcified blood vessels).



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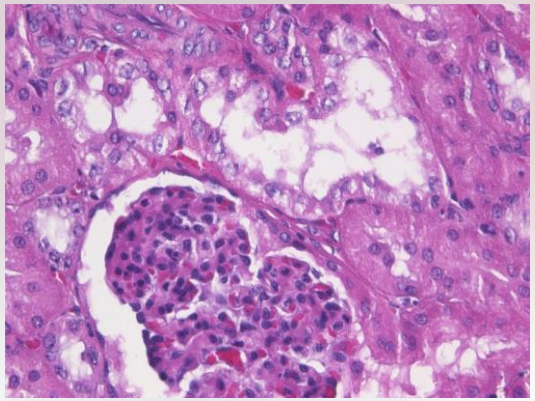
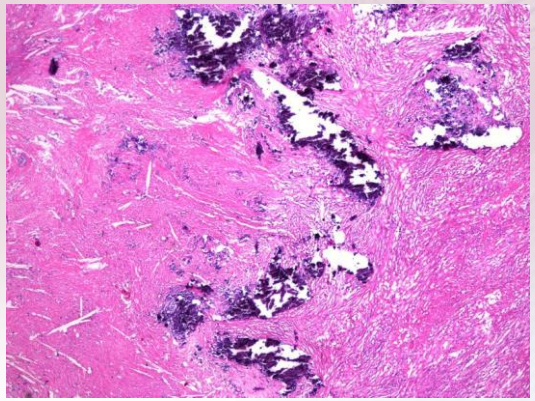
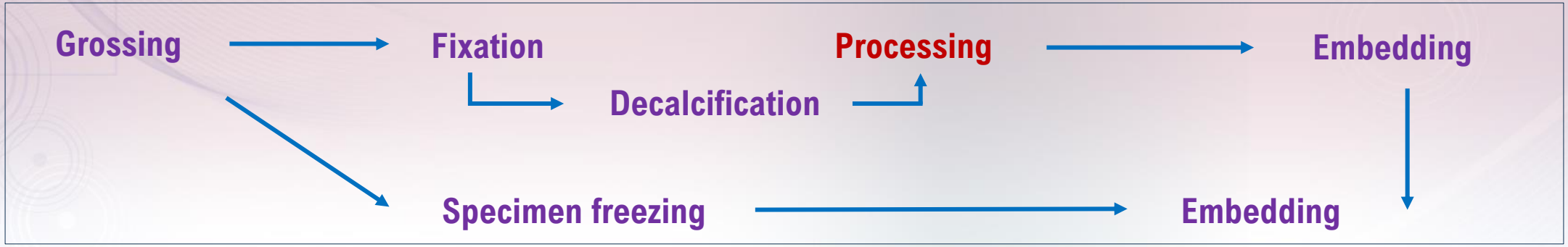
Strong acids	Rapid decalcifier based on hydrochloric or nitric acid
Weak acids	Slow but gentle decalcifier based on formic acid, often combined with formalin
Chelating agents	Chelating agents, such as EDTA, work very slowly by capturing and reducing calcium ions





PROCESSING

- Use correct processing times according to tissue size and type; under processed samples make sectioning impossible, over processed tissue lose its morphology integrity making them inadequate for proper diagnosis.
- Use optimal reagents
  - reagent alcohols, ideally in gradually increased concentrations.
  - when using xylene substitutes, increase processing times and rotate more often.
  - paraffins, ideally infiltrating type with limited amount of plasticizers.
- If possible, employ agitation on tissue processor. Use vacuum to aid reagent infiltration.
- Select right cassettes:
  - pore size depending on specimen size.
  - validated for use on automated printers.



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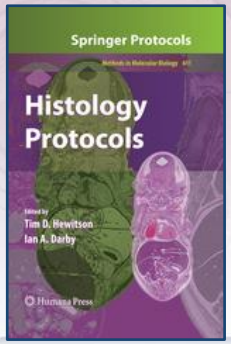
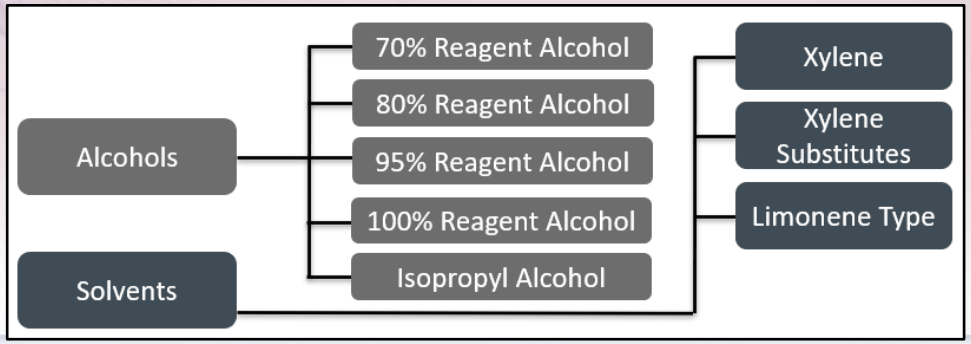
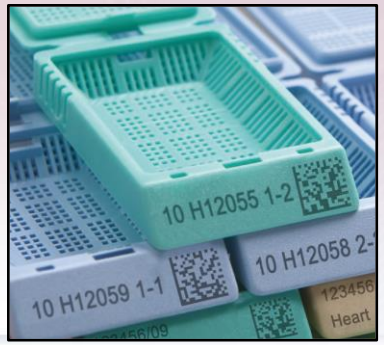
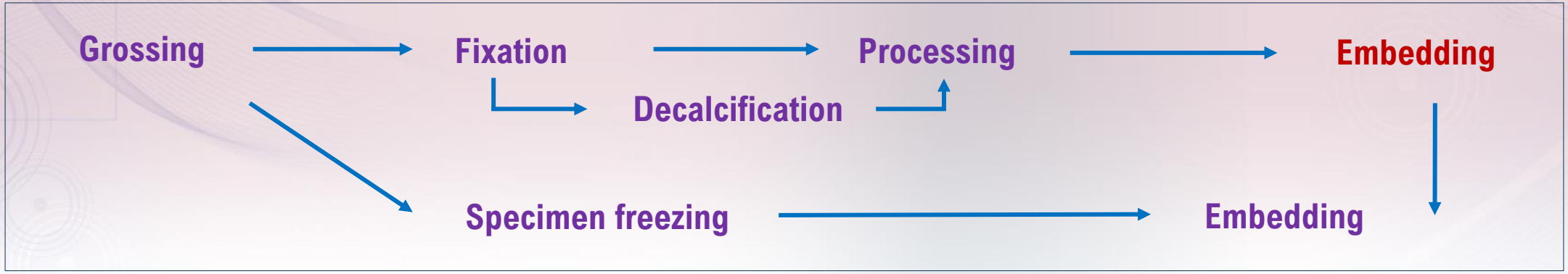






EMBEDDING

- Use the right embedding technique:
  - proper sample orientation (e.g., elongated specimens, rigid or fibrous samples).
  - optimal molds (size, type).
  - avoid introducing air bubbles when making blocks.
- Monitor temperature of forceps, hot plate (heat damage) and cold plate (cracking paraffin) to avoid artifacts.
- Do not overfill or underfill mold with paraffin.
- Choose best embedding media:
  - paraffins with high polymer content provide better structural support.
  - containing dye for better visualization of small or opaque samples.



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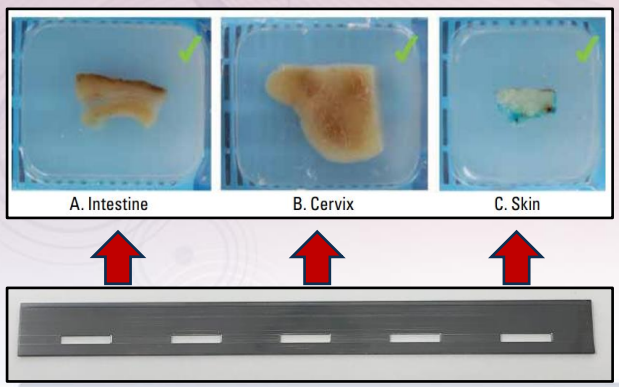
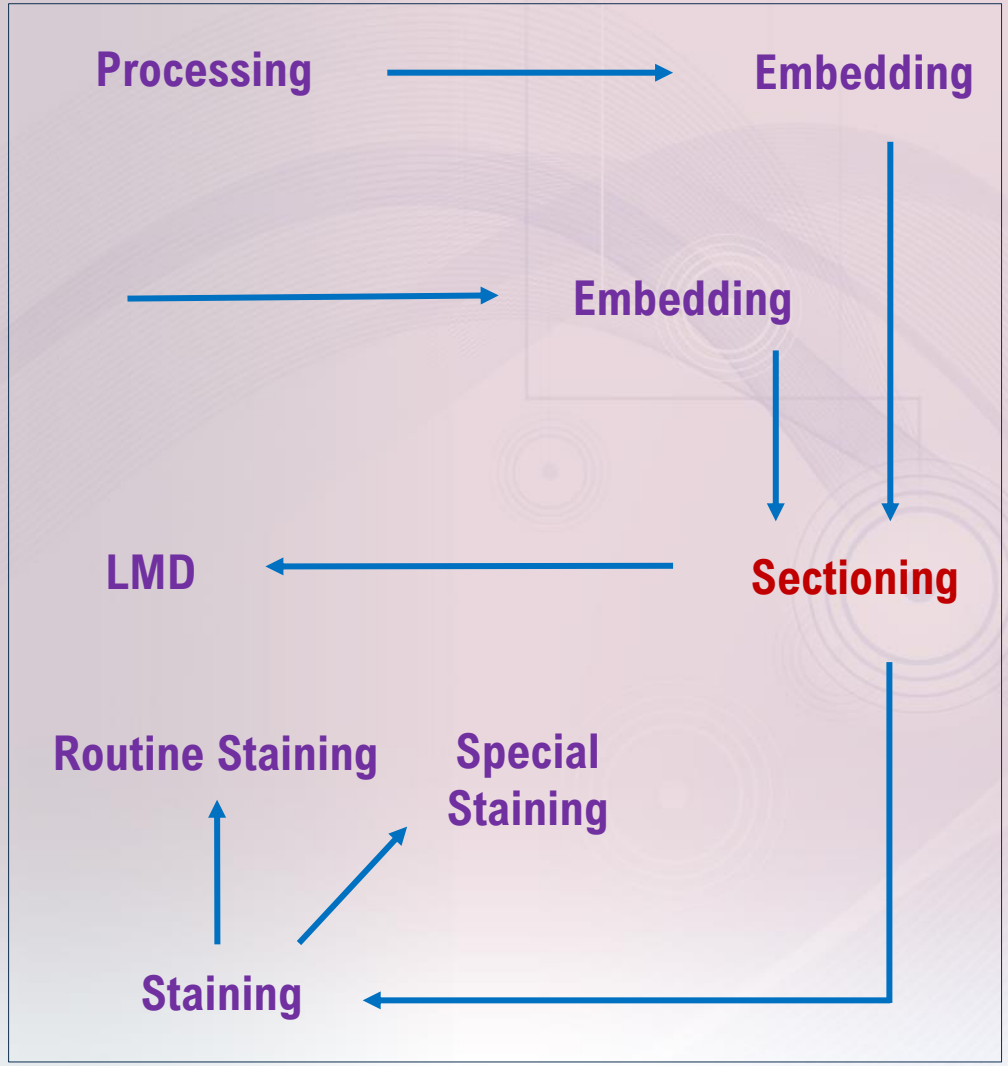


PARAFFIN SECTIONING

- Always choose high quality blade:
  - consider using high profile for dense, hard samples.
  - low profile blade for soft and biopsy sized specimen.
- Optimized clearance angle (tilt angle) for each microtome and blade type, trim blocks carefully (at 20µ increments).
- To avoid compression, chill carefully trimmed blocks on cold wet surface.
- Monitor flotation bath temperature to avoid over-expansion and separation.
- Tighten all clamps (blade clamp, knife holder base, knife tilt, chuck orienting, clearance angle) to prevent microtome vibrations or artifacts are created
- Adhesive (coated) slides are strongly recommended when sectioning difficult tissues or sectioning for IHC/ISH/Special Stains procedures to prevent section detaching or lifting.
- Use water bath additives properly: don't use them on coated slides!



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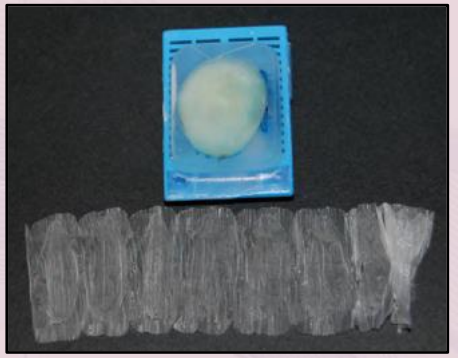

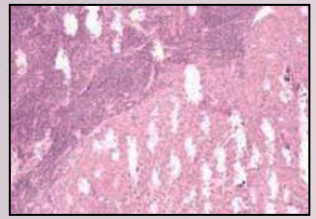

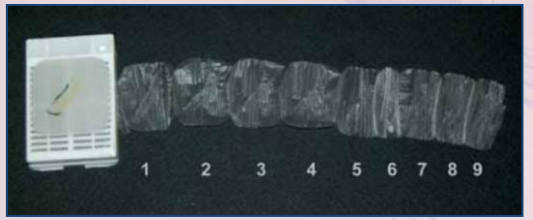

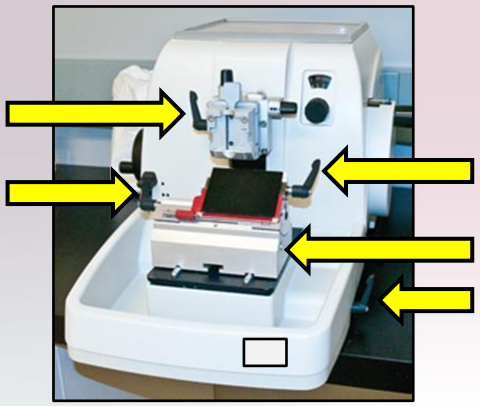

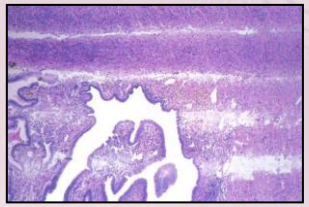

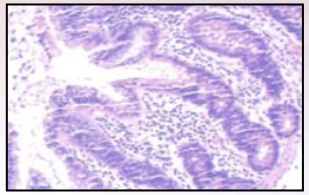

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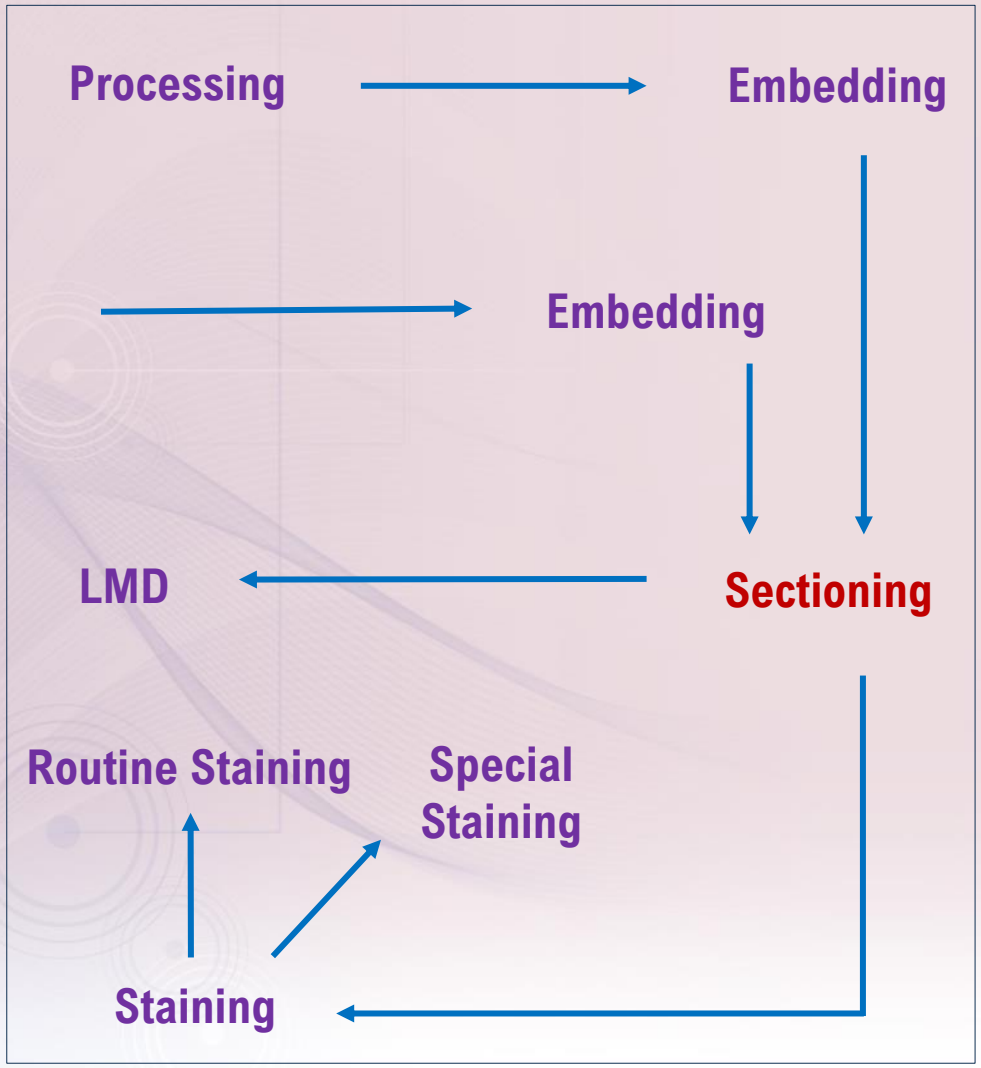

- Chose right blade for tissue type:
  - try high profile for dense, hard samples.
- Since you are sectioning non-fixed samples, often thicker than paraffin sections, adhesive (coated) slides are strongly recommended to prevent section detaching or lifting.
- Create frozen blocks air-bubble free using proper technique and best quality frozen section compound.

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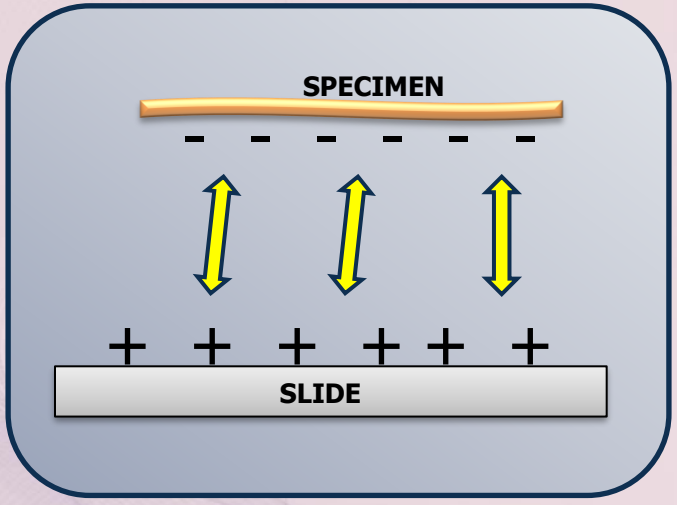

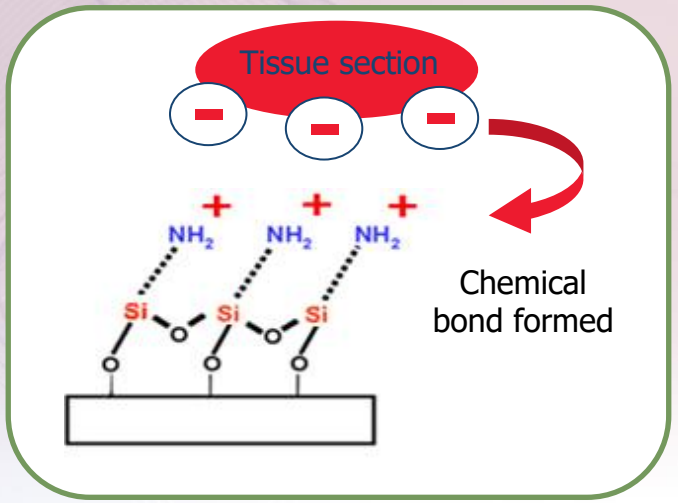

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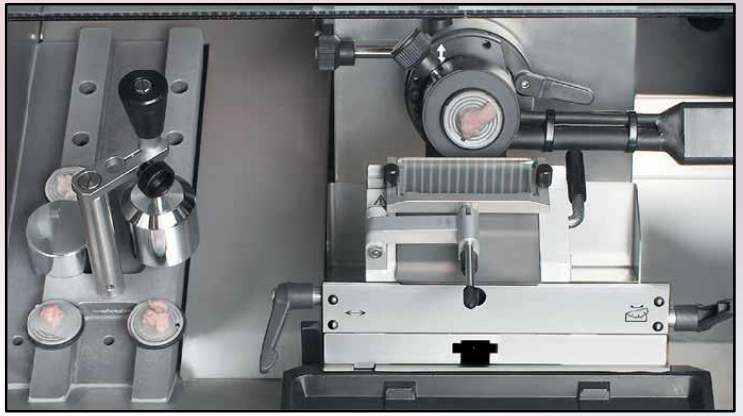
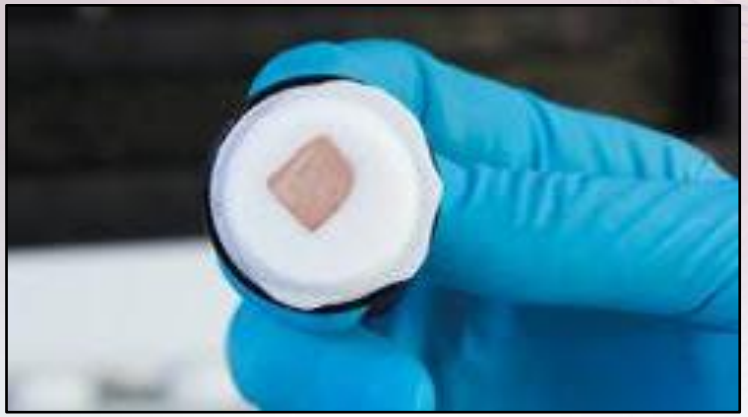
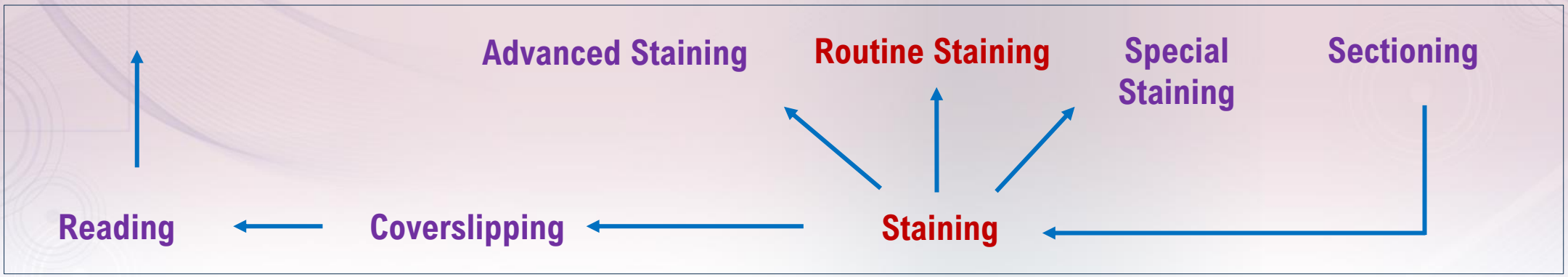




ROUTINE STAINING

- Before staining, drain slides vertically, then dry at approx. 60-70˚C. Higher temperature might cause sections to crack.
- Optimize all staining steps, their exact times and agitation speed. Use control slides regularly to monitor stain quality. Do not alter validated, close staining systems times unless allowed.
- Muddy hematoxylin, pale eosin staining are signs of deteriorating dyes.
- Unstained or uneven staining are signs of poor management of ancillary reagents rotation/replacement.
- Chose adequate differentiator for staining type: progressive – weak acid, regressive – strong acid.
- When using xylene substitutes, increase staining times and rotate/replace more often.



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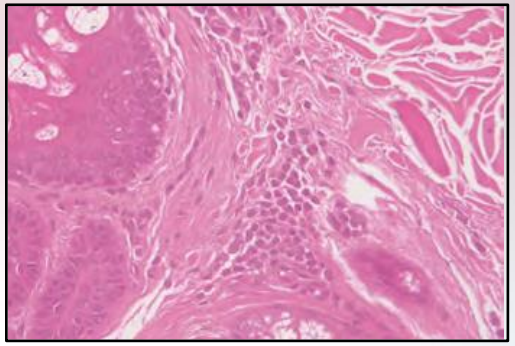
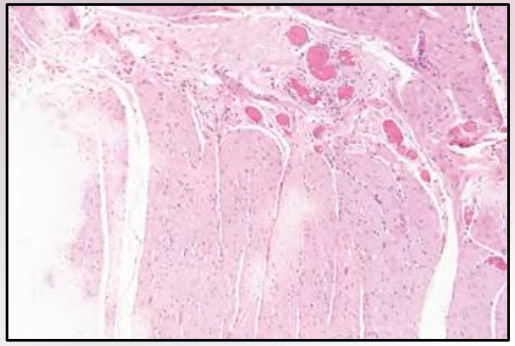
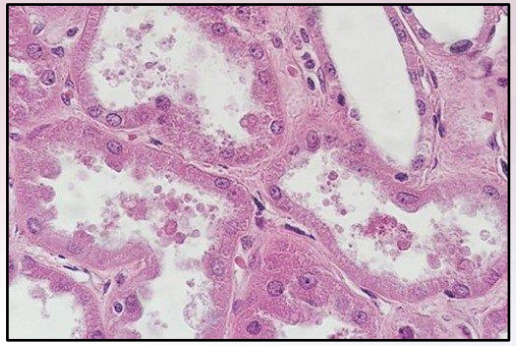
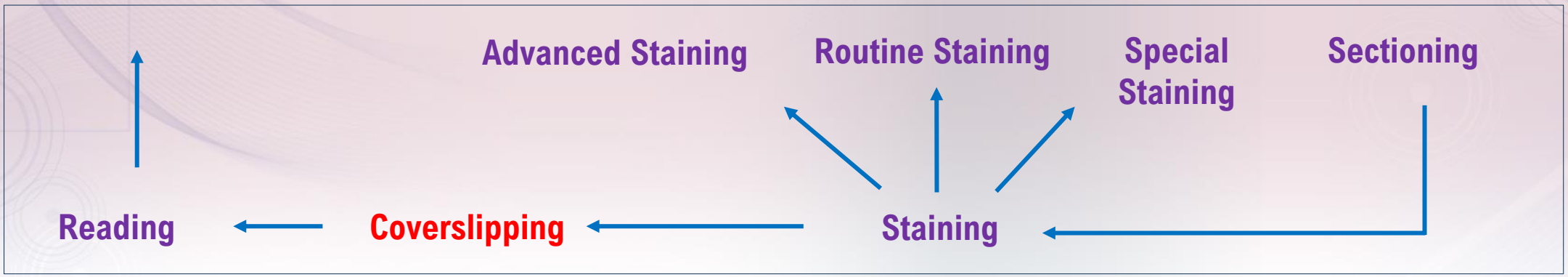




COVERSLIPPING

- When using automated coverslipper always use premium cover glass to avoid glass sticking (double coverslipping) and instrument jamming (slides drying).
- When changing mounting media (type, brand) always adjust instrument settings for appropriate viscosity; changing needle might be also necessary.
- When filling the coverslipper reservoir with fresh media, let it aerate to avoid bubbles or air pockets under the coverglass. Before each use, follow instrument priming steps to eliminate the air that might be trapped inside the lines.
- Use proper cover glass size to avoid covering painted part of slides and introducing air pockets.
- Ideally, solvent in a coverslipper bath reservoir should be the same as solvent on a stainer for maximum compatibility.
- Use mounting media that contain an anti-oxidant to avoid fading of the stained slides.



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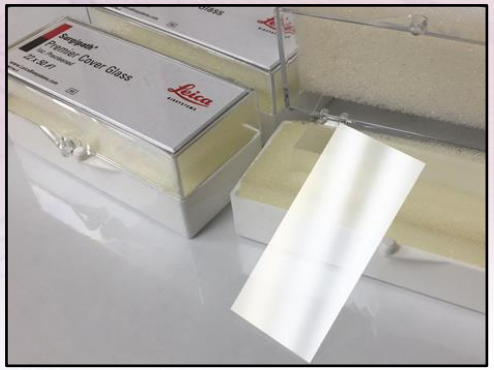
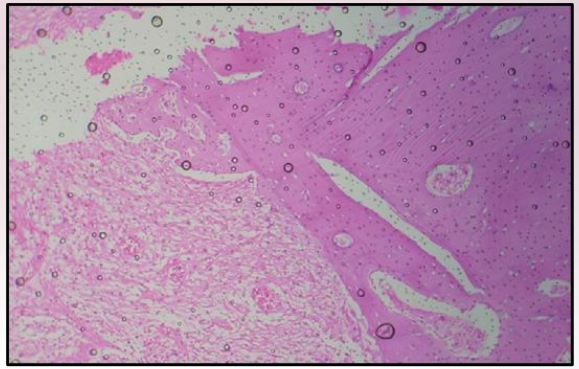
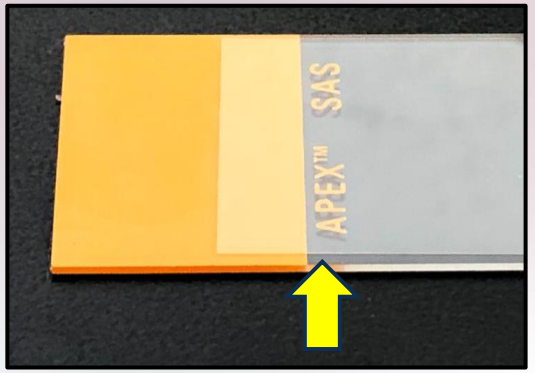
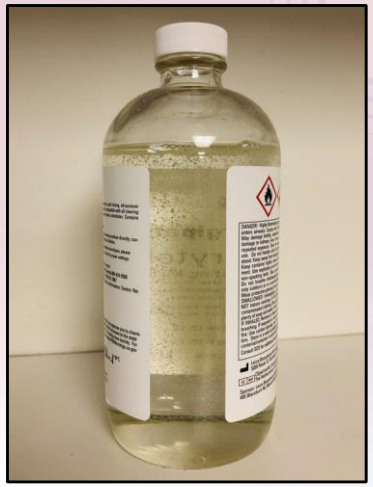


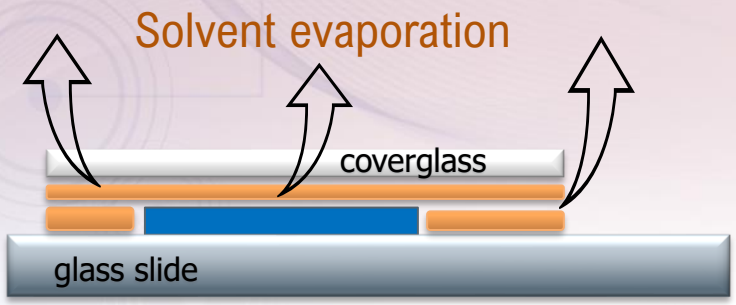


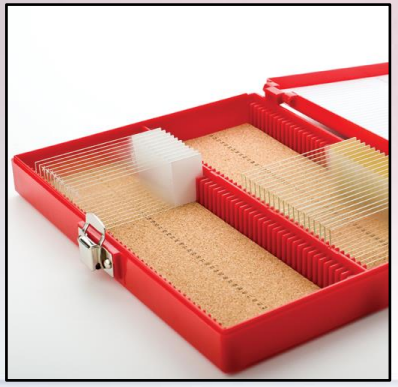


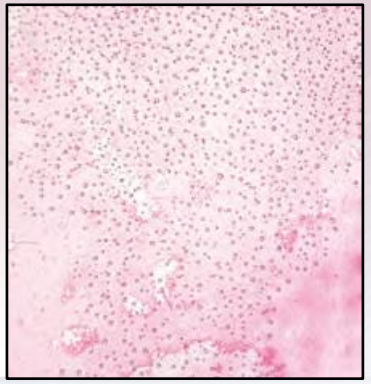


STORING AND ARCHIVING

- Depending on the mounting media type, most are ready to be handled and **viewed** within minutes.
- Once slides are coverslipped they still, however, need additional time to be stored.
- Mounting media completely solidifies after **48-72h** and only then slides can be stacked for **archiving or shipping**.
  If not enough time is allowed, mounting media leaking from under the coverslips will cause adjacent slides to glue or stick together.
- Boxing slides too soon will cause the same effect – mounting media will adhere then to the bottom of the box.
- Although good quality mounting media contains an anti-fading agent, never store slides on open flat boards in direct sunlight.
- The long-term storage qualities of the mountant must be known because crystals can appear in poor quality mountant, sometimes after a long period (months or years).







SUMMARY

- Understanding all histology steps, science and choices of consumable products will:
  - Minimize repeated work
  - Decrease time and laboratory costs
  - Optimize workflow
  - Improve performance in patient care

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