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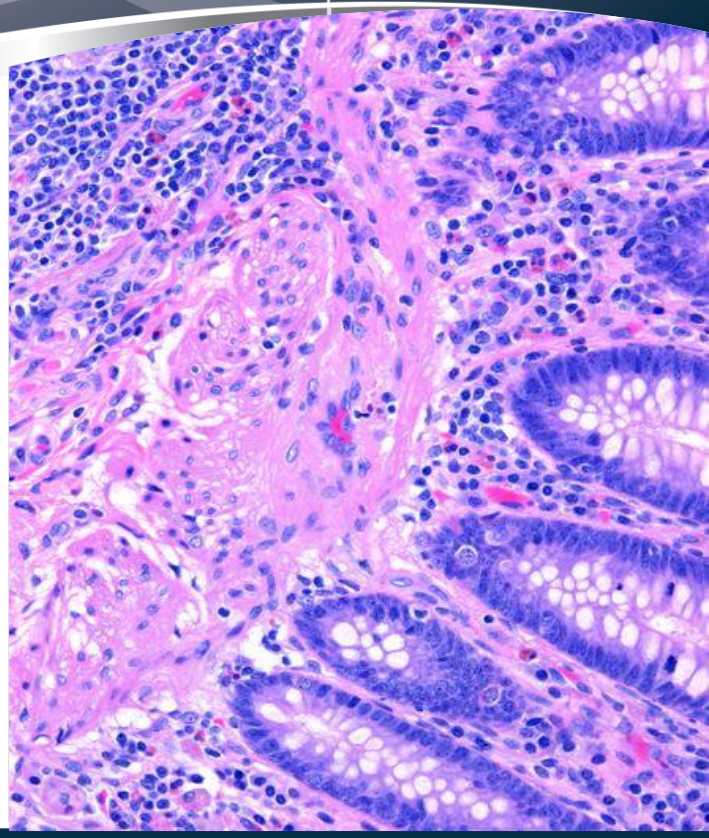

# Science of Tissue Processing

**Andrew Lisowski, M.S., HTL (A.S.C.P.)**



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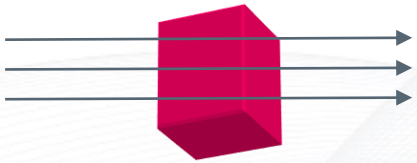
# What is Tissue Processing?

- Tissue processing is a procedure of removing water from cells and replacing it with a medium which solidifies allowing thin sections to be cut on a microtome.
- Once tissue is properly fixed it goes through a process which involves the following steps:
  - Dehydration
  - Clearing
  - Infiltration
- Tissue processing is routinely done on an instrument called Tissue Processor.

# Tissue Processing - Overview

- "Tissue processing" describes the steps required to take animal or human tissue from fixation to the state of complete infiltration with a histological paraffin.
- Subsequently, the processed tissue is made into a paraffin block so it can be sectioned on the microtome.

**Step 1:** Alcohol

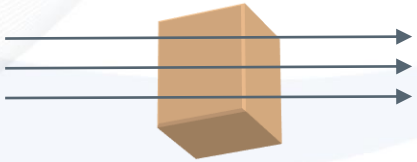


Alcohol replaces water in all cells

**Step 2:** Xylene

Xylene dissolves alcohol

**Step 3:** Paraffin



Paraffin displaces xylene. Specimen is now ready to be embedded.

Specimen

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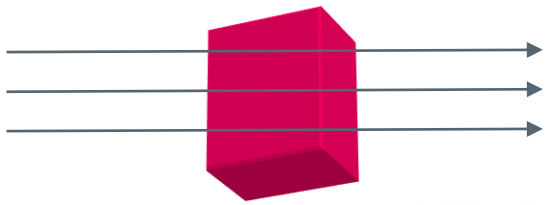
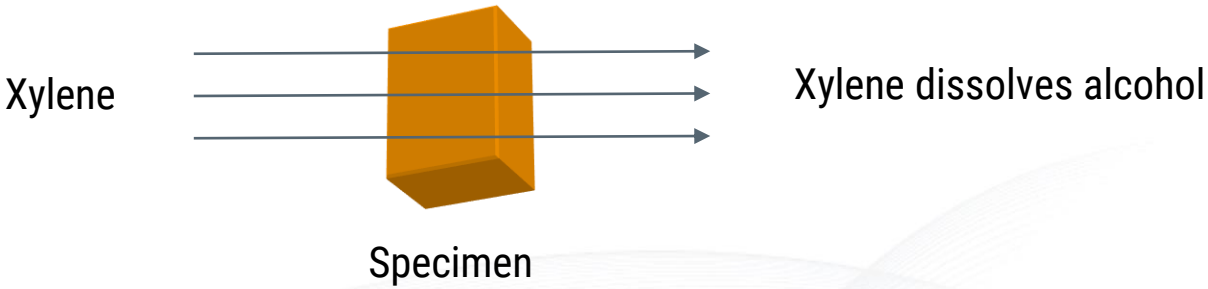
# Step 1: Dehydration



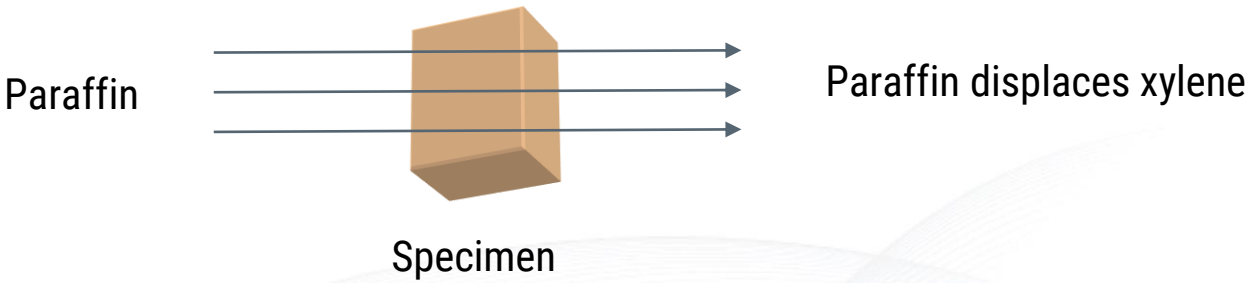

- Since paraffin is hydrophobic (immiscible i.e., not mixable with water), water inside a specimen must be removed before it can be infiltrated with paraffin. This process is carried out by immersing specimens in a series of alcohol.
- Alcohol progressively replaces water in all the cells of the specimen.
- A series of increasing (typically from 70% to 100%) alcohol concentrations are used to avoid excessive distortion of the tissue.

# Step 2: Clearing



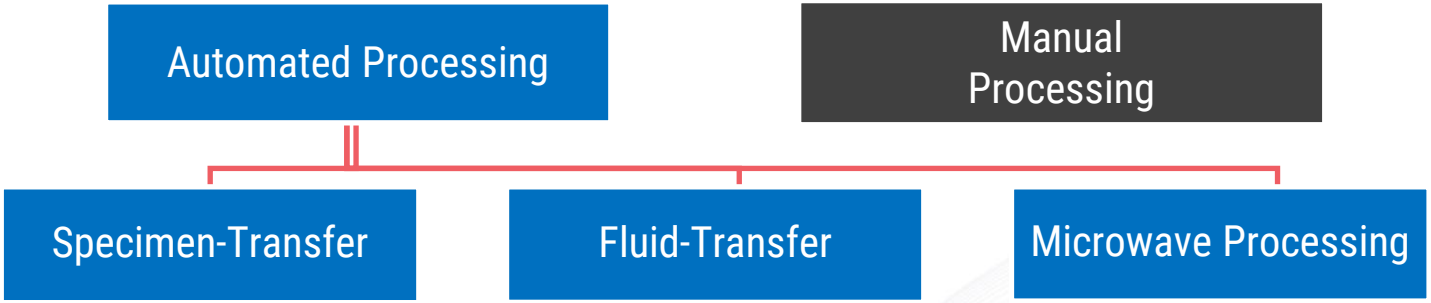

- Since alcohols and paraffins are not miscible, an intermediate solvent that is fully miscible with both (such as xylene), must be used.
- This solvent displaces the alcohol in the tissue through the process called “clearing”.
- “Clearing” relates to how clearing agents impart an optical clarity or transparency to the tissue due to their relatively high refractive index.
- Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to paraffin infiltration.
- To make sure that all traces of alcohols are removed from tissues being processed, multiple changes of fresh, clear of carried-over alcohol, are required.

# Step 3: Infiltration




- The specimen can now be infiltrated with paraffin. Molten paraffin infiltrates tissues and when cooled solidifies to a consistency that allows sectioning on a microtome.
- The amount of structural support given by solidified paraffin can be regulated by choosing different paraffin formulations.
- Multiple changes of histological paraffin are required to completely displace the clearing agent.
- Paraffin infiltration is greatly enhanced by vacuum.

# Processing Methods

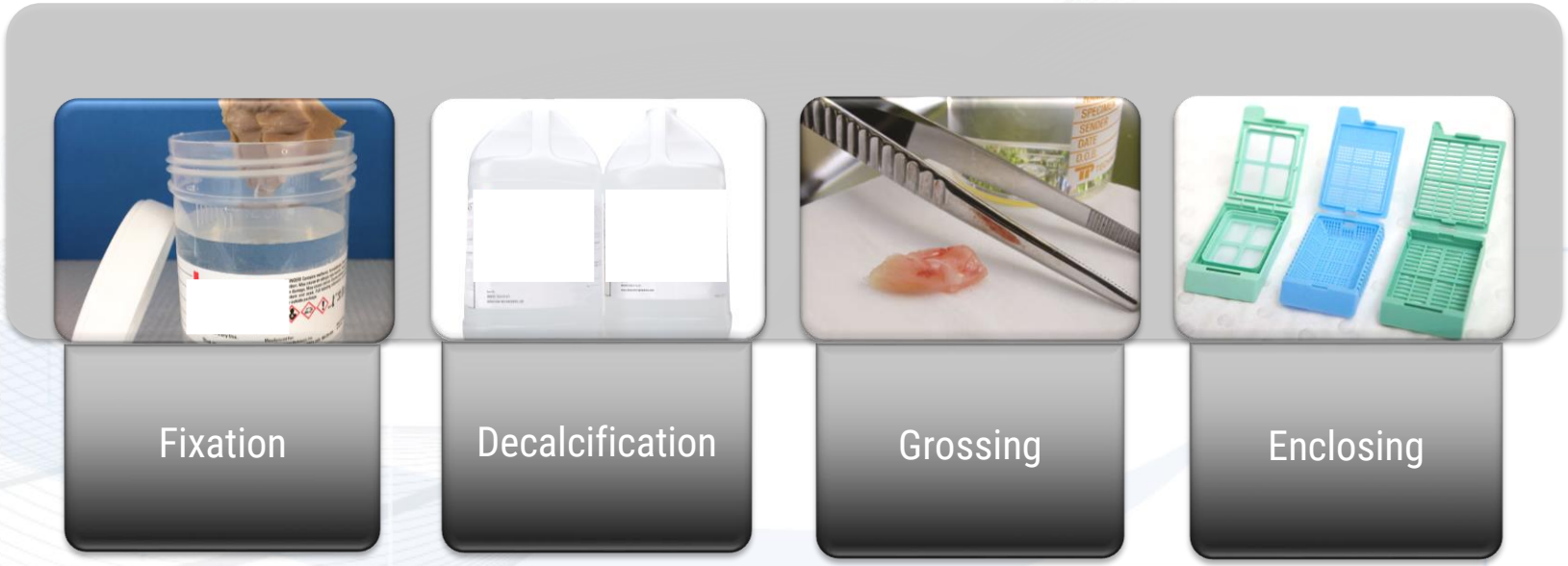



- Manual Processing
  - Slow, most labor-intensive method since transfer of specimens or changing reagents is done by hand. With an advance of automation this method is almost obsolete.
- Automated Processing
  - Specimen-transfer or "dip and dunk" processors: instruments which transfer cassettes from station to station in a rotary or linear configuration.
  - Fluid-transfer or "enclosed" instruments hold the specimens in a process chamber or retort and the reagents are pumped in and out during processing.
  - Microwave assisted processing: might require manual transfer of specimen or reagents, it accelerates processing by heating reagents.

**Most modern fluid-transfer processors employ raised temperatures, effective fluid circulation and incorporate vacuum/pressure cycles to enhance processing and reduce processing times.**

# Preliminary Steps for Optimal Processing

Steps leading to the processing stage are crucial for obtaining morphological and histochemical information from the specimen.

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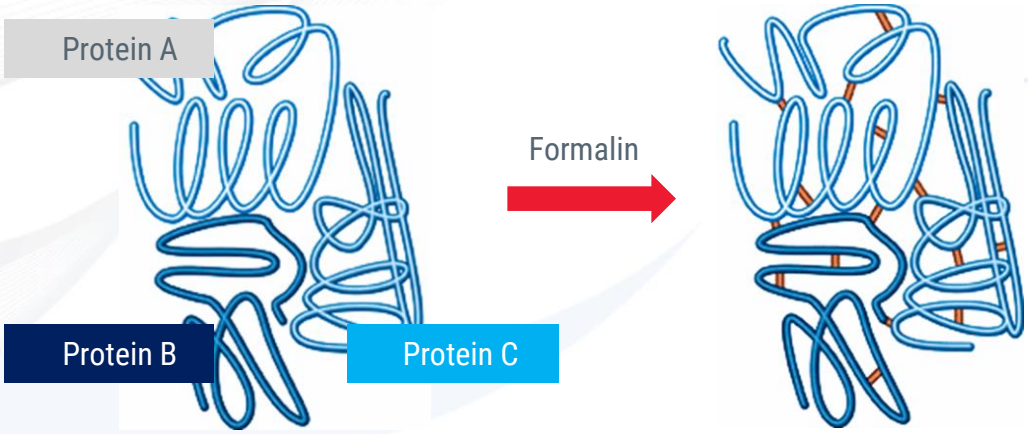
# Preliminary Steps: Fixation

- Fixation is a critical step in the preparation of histological sections. If it is not carried out under optimal conditions or if fixation is delayed, a tissue specimen can be irreversibly damaged compromising morphological and histochemical information.
- The optimal time for fixation will vary between fixatives, tissue type and size.
  Dense or fatty tissues usually require more time to be fully fixed.
- Most frequently the routine fixative will be neutral buffered formalin. It can be incorporated into the processing schedule on enclosed tissue processors.

Formalin is by far the most popular fixative reagent used in histopathology. It fixes tissue by immobilizing proteins creating an extensive matrix of cross-links.



# Preliminary Steps: Decalcification

- Bone and other calcified specimens must be decalcified prior to processing and paraffin infiltration.
- Once the mineral has been removed, a standard processing schedule can be used.
- Most of the acid decalcifier should be washed away before processing to avoid contaminating the processing reagents.
- Despite complete decalcification, bone (particularly compact bone), will contain dense areas that require thorough processing. In such case choosing a longer schedule is advised.

# Preliminary Steps: Grossing

- For optimal fixation and subsequent high-quality processing, the dimensions of tissue specimens are important. Ideally the thickness should not exceed 4mm and the specimen should fit into a histology cassette without distortion.
- It is possible to “over-process” a small and delicate specimen or “under-process” large, dense specimens making them very difficult, if not possible, to section on a microtome.
- Processing times are also different for different tissue types: some tissues are penetrated by reagents relatively easily (kidney, lung) while others are much more resistant (cervix, muscle) and require more time, i.e., different schedule.

# Preliminary Steps: Enclosing Specimen

- Cassettes hold and protect the specimen while it undergoes processing.
- Cassettes chosen must be completely resistant to the solvents and heat used in processing. They must not distort during use so that there is no chance that the specimen will escape into the processing reagents.
- Perforated cassette surfaces must allow for adequate fluid exchange and proper drainage.
- Small specimens can be protected by wrapping them in fine, lint-free papers, placing them in fine-mesh biopsy bags or “sandwiched” by using biopsy pads.

# Processing Reagents: Fixatives

- In an ideal situation every specimen would be thoroughly fixed before processing is commenced.
- Small specimens that do not require long fixation times are often exclusively fixed on a processor ("on-line fixation").
- Most laboratories will include fixative as a first step on their processor so as to provide some additional time in fixative before proceeding with dehydration.

# Processing Reagents: Dehydrants

- Most dehydrating reagents are alcohols (i.e., ethanol, methanol, isopropanol, butanol).
- Ethanol, the most widely used dehydrant, is a drinking alcohol and hence strictly controlled (license and recordkeeping required) by most governments. To make it less troublesome, manufactures add methanol and/or isopropanol to make it unfit for human consumption. Such product is known as reagent or denatured alcohol and is not controlled.
- Dehydration process frequently begun with 70% alcohol, followed by several changes of each alcohol with gradually increased concentrations, usually 80%, 95% and 100% alcohol.

# Processing Reagents: Dehydrants

- Agitation
  - Mechanical agitation does increase the speed at which alcohol replaces the water.
- Heat
  - Tissue processors permit gentle heat to be applied during dehydration. Since warming fluids makes them less viscous, it increases the effectiveness of dehydration by increasing the ability to penetrate the tissue.
- Time
  - Long, slow dehydration gives the best quality results. Overnight processing is probably the most common, with significant numbers of laboratories using same day processing for small biopsies.
- Shrinkage
  - Dehydration, when done at room temperature, starting with moderate concentrations of alcohol, causes a little tissue shrinkage.

# Processing Reagents: Clearing Agents

- The process of clearing was originally termed as such because the reagents used for this step have a high index of refraction and will render tissue transparent.
- Clearing agents are solvents that are fully miscible with both alcohol and paraffin. They displace the alcohol in the tissue, then this in turn, will be displaced by molten paraffins in the next step.
- Prolonged time in many of the clearing agents will produce hard, brittle tissue while inadequate clearing will make tissue extremely difficult to section.

# Processing Reagents: Clearing Agents

- Number of changes
  - Since the tolerance level of paraffin for clearant is greater than it is for water, fewer changes are required than for dehydration. An absolute minimum of two changes should be used, but three or four are recommended.
- Agitation
  - As with dehydration, gentle agitation can improve clearing and reduce the time necessary for it to be done.
- Time
  - This varies depending on the particular clearing agent used. Aromatic compounds (xylene, toluene, benzene) have greater tolerance for water than aliphatic compounds (xylene substitute, limonene) therefore require less changes and less time to replace alcohol.

# Choosing Clearing Agents


- Xylene
  - An aromatic compound, is probably the most popular of clearing agents even though many laboratories are looking to use less toxic substitutes. It displaces alcohol quickly from tissues and in turn, being an excellent paraffin solvent itself, can be displaced from tissues relatively easily by melted paraffin.
- Xylene Substitutes
  - Alkane and limonene-based solvents have become widely available for use as clearing agents. They have a lower toxicity than xylene and consist of mixtures of aliphatic hydrocarbons. These reagents, however, have an intolerance for water and processing schedules need to be extended.

# Processing Reagents: Paraffins

- Paraffin heated to an approximately 60°C becomes a liquid and can infiltrate tissues. After several infiltration steps it is allowed to cool to a room temperature where it solidifies to a  consistency that allows for sectioning.
- Since solidified paraffin holds the cells and intercellular structures in their proper relationship it is also referred to as  supporting medium.
- Paraffins are mixtures of purified wax and various additives that may include plastic resins (polymers), antioxidants, dyes and other additives.
- Specific chemical composition defines paraffin characteristics. By manipulating the ratio of components or using different chemicals we can change product properties, like melting point or hardness.

# Processing Reagents: Paraffins

- Large, fatty, or dense tissues are processed on a longer cycle than small and delicate specimens.
- Paraffin infiltration is greatly aided by vacuum; however vacuum and heat should be applied cautiously when processing very small specimens.
- When using modern enclosed tissue processors, it is recommended that at least three changes of paraffin are used to make sure that all traces of clearing agent are removed from tissues being processed.

# Xylene-Free Processing

- Since xylene is a relatively hazardous solvent, laboratories are under pressure to seek less toxic alternatives for routine use.
- A xylene-free method has been developed that excludes xylene, not only as the processing step, but also for de-paraffinizing steps during routine staining.

| Fixation |
| --- |
| Ethanol based dehydration |
| Clearing with Xylene |
| Paraffin Infiltration |

**Conventional processing steps**

| Fixation |
| --- |
| Ethanol based dehydration |
| 80/20 mixture: ethanol/isopropanol dehydration |
| Absolute isopropanol dehydration |
| Paraffin Infiltration |

**Xylene-free processing steps**

Despite being classified as hazardous chemical, xylene as one of the most efficient solvents, is the most popular solvent in a histology laboratory.

# Microwave Processing

- Within the last decade, tissue processing with the microwave oven has been introduced into histology laboratories.
- Processing times are significantly reduced but throughput is very low.
- Only laboratory microwave ovens should be used as the temperature must be carefully controlled and the microwave must be vented just like a chemical fume hood.
- This technology is especially useful for biopsy sized specimens.
- Regents used in microwave processing include ethyl alcohol, isopropyl alcohol and paraffin.

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