



HISTOPATHOLOGIC & CYTOLOGIC TECHNIQUES LEC

Module 6: Conventional Tissue Processing with Automation

MTHC31-24

Prof. Neil Allen Elquinto

TOPIC OUTLINE

- I. Tissue Processing
 - A. Overview of the Steps in the Tissue Processing for Paraffin Sections
- II. Automatic Tissue Processing
- III. Factors that Impact the Duration of Tissue Processing and Extent of Infiltration
- IV. Technical Considerations

TISSUE PROCESSING

Describes the various steps required to take the tissue from fixation to the state where it is completely infiltrated with suitable histological wax and can be embedded ready for section cutting on the microtome.

In order to enable the pathologist to diagnose the presence or absence of disease, the histotechnologist needs to produce a tissue section of good quality that allows for adequate interpretation of microscopic cellular changes. Solid tissues need to be fixed and processed to preserve their structures, and eventually impregnated with an appropriate hardening substance to permit making thin slices suitable for staining and microscopic evaluation.

Processing solid structures and tissues in the following order:

- 1. Numbering and Gross/Macroscopic Examination
- 2. Fixation
- 3. Decalcification (optional)
- 4. Dehydration
- 5. Clearing
- 6. Impregnation (Infiltration)
- 7. Embedding
- 8. Trimming
- 9. Section – Cutting (Microtomy)
- 10. Staining
- 11. Mounting
- 12. Labeling

Overview of the Steps in the Tissue Processing for Paraffin Sections

Step	Description
Numbering and Gross / Macroscopic Examination	➤ First and most important step in Histopathology ➤ Upon the receipt of organs, tissues and even fluids in the laboratory, Histopathological Request is required, filled up with complete patient information such as Name, Age, Sex, Birthday, Clinical Diagnosis, Procedure done to the patient (ex. Appendectomy), Laterality of the organs (if applicable) etc. ➤ Identify properly all the specimens received without the need of writing

	the patient's name or accompanying specimen tag. ➤ Entering the details of the specimen in a log book. ➤ In numbering, the specimen number is preceded by either S (Surgical), A (autopsy) or C (cytology). The year is also indicated. ➤ Example: S2020-0345 S means surgical, 2020 means year indicated, 0345 means the specimen number. ➤ After numbering, the pathologist will describe the gross description of the specimen. The Medical Technologist will then write down the description at the back of the request. ➤ Specimen size for processing: 3 x 2 x 0.5 cm and 3-5 mm thick.												
Fixation	➤ It is important that tissues are handled carefully and appropriately fixed as soon as possible after arriving in the laboratory. ➤ The specimen is placed in a liquid fixing agent (fixative) such as formalin. This will slowly penetrate the tissue causing chemical and physical changes that will harden and preserve the tissue and protect it against subsequent processing steps. ➤ Fixation time – it is the period wherein the fixative completely penetrates the tissue that allows the chemical reactions of fixations to reach equilibrium. ➤ One of the critical steps in the preparation of histological sections. If the fixation of tissues were delayed or it does not meet the criteria and optimum conditions it can be irreversibly damaged. ➤ Formalin-fixed, paraffin embedded tissues may be stored indefinitely at room temperature, and nucleic acids (both DNA and RNA) may be recovered from them decades after fixation.												
Dehydration	➤ Most of the water in a specimen must be removed before it can be infiltrated with wax. ➤ This process is commonly carried out by immersing specimens in a series of ethanol (alcohol) solutions with increasing concentration to avoid excessive distortion of tissue until water-free tissue is reached. ➤ Typical dehydration sequence for specimens not more than 4mm thick would be: <table><tr><td>70% ethanol</td><td>15 mins.</td></tr><tr><td>90% ethanol</td><td>15 mins.</td></tr><tr><td>100% ethanol</td><td>15 mins.</td></tr><tr><td>100% ethanol</td><td>15 mins.</td></tr><tr><td>100% ethanol</td><td>30 mins.</td></tr><tr><td>100% ethanol</td><td>45 mins.</td></tr></table> ➤ Ethanol is a poor fat solvent. To ensure complete dehydration, a superior fat solvent such as acetone or isopropanol should be added before the final absolute ethanol, and chloroform or trichloroethane used as the transition solvent.	70% ethanol	15 mins.	90% ethanol	15 mins.	100% ethanol	15 mins.	100% ethanol	15 mins.	100% ethanol	30 mins.	100% ethanol	45 mins.
70% ethanol	15 mins.												
90% ethanol	15 mins.												
100% ethanol	15 mins.												
100% ethanol	15 mins.												
100% ethanol	30 mins.												
100% ethanol	45 mins.												



HISTOPATHOLOGIC & CYTOLOGIC TECHNIQUES LEC

Module 6: Conventional Tissue Processing with Automation

MTHC31-24

Prof. Neil Allen Elquinto

Clearing	➤ After the tissue is dehydrated, it is transferred to an intermediate solvent that is fully miscible with both ethanol and paraffin wax. ➤ The term “clearing” has been chosen because many (but not all) 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 wax infiltration. The tissues is immersed to the three changes of xylene to replace the ethanol after dehydration, ➤ Xylene will be replaced by the molten paraffin for infiltration. ➤ Typical clearing sequence for specimens not more that 4mm thick would be:	
	Xylene	20 mins.
	Xylene	20 mins.
	Xylene	45 mins.
Infiltration	➤ The cleared tissue is infiltrated with a suitable histological wax (usually paraffin) which is liquid at 60°C and then allowed to cool to 20°C in order into a consistency that allows sections to be cut. ➤ These waxes are mixtures of purified paraffin wax and various additives that may include resins such as styrene or polyethylene that allow them to be sectioned thin enough to a microtome, forming ribbons that can flatten fully when floated on a warm water bath. ➤ To completely displace the clearing agent, a typical paraffin infiltration sequence or paraffin for specimens not more than 4mm thick would be:	
	Paraffin wax	30 mins.
	Paraffin wax	30 mins.
	Paraffin wax	45 mins.
Embedding	➤ It is orienting the tissue on a mold then the molten paraffin wax will pour to form a solid block. ➤ The infiltrated tissue is removed from the cassette and very carefully oriented in a suitably sized metal mold so that the “plate of section” can be determined. ➤ Correct orientation of the tissue in a mold is the most important step in embedding. ➤ Incorrect placement of tissues may result in diagnostically important tissue elements being missed or damaged during microtomy. Usually tissues are embedded with the surface to be cut facing down in the mold. ➤ The main part of the labeled cassette is placed on top of the mold and is ready to be sectioned in a microtome. ➤ Double embedding is the process by which tissues are first embedded of fully infiltrated with a supporting medium such as agar or nitrocellulose, then infiltrated a second time with wax in which they are also embedded.	

	➤ Double embedding in agar-paraffin is a reliable and convenient method of handling minute and friable tissue fragments such as curetting and endoscopic biopsies, which can be lost during tissue processing. ➤ Correct orientation of tissues in embedding is very crucial particularly if the tissue slides are subjected for histochemistry and immunohistochemistry. ➤ Tissues may shrink by the time they are infiltrated with wax, but if adequately processed, they will still show good morphological detail and allow for accurate histopathological evaluation.	
Section-cutting	➤ This is done with a microtome. Good microtomy techniques will minimize artifacts that can lead to difficult diagnostic interpretation of special stains. ➤ Specimens for Routine Hematoxylin and Eosin stain (H&E) are cut 3-5 μm in thickness. Tissues to be examined for amyloid deposits are better sectioned 8-12μm, whereas kidney biopsies should be cut at 2μm for optimal viewing of the structures of glomeruli. ➤ Paraffin wax is routinely used however, Epoxy resins are the most commonly employed embedding media for semi-thin and ultrathin sections, but acrylic resins are also used, particularly where immunohistochemistry is required. ➤ Thicker sections (0.35μm to 5μm) of resin-embedded tissue can also be cut for light microscopy. ➤ The microtome is nothing more than a knife with a mechanism or advancing a paraffin block across the knife. Knives are either of the standard thick metal variety or thin disposable variety (like a disposable razor blade). Usually this distance can be set, for most paraffin embedded tissues to 6 to 8 microns. ➤ Plastic blocks (methacrylate, araldite, or epon) are sectioned with glass or diamond knives that can cut sections down to about 1 mm. ➤ Thin sections for electron microscopy (0.25 micron) are best done with a diamond knife. ➤ It is important to have a properly fixed- and embedded-specimen or much artifacts can be introduced during the sectioning. Common artifacts include tearing, ripping, creases, holes or folding of sections, etc.	
Mounting	➤ Paraffinized ribbons of serial tissue sections can be removed from the microtome knife as they are cut, by using a wooden tongue depressor blade. ➤ In this process, a slight traction is exerted on the end of the ribbon, stretching it gradually over the wooden blade while floating in a warm water bath.	



HISTOPATHOLOGIC & CYTOLOGIC TECHNIQUES LEC

Module 6: Conventional Tissue Processing with Automation

MTHC31-24

Prof. Neil Allen Elquinto

	➤ The temperature should be kept warm bath should be kept at 5-10°C below the melting point of the embedding wax. The ribbon must not be let in the bath for more than 1 or 2 minutes, or over-hydration of the tissue will be produced, simulating the appearance of edema fluid when examined microscopically. ➤ One of the most serious issues faced in histotechnology is misidentification of tissues, which includes mislabeled specimens, block identification problems, and tissue contaminants. ➤ A potentially dangerous mistake that can take place when mounting sections from floating baths is the “shedding” of friable small tissue fragments that float freely on the surface of the water, which can be picked up when mounting the slides. ➤ “Floaters” – tiny pieces of unrelated tissue commonly cause problems in interpretation of the biopsies by the pathologists that might cause false results. ➤ Meticulously cleaning of the microtome water bath and frequent clearing and changing of the water will alleviate the danger of rare contaminants being carried over to subsequent sections being mounted.
Staining	➤ The tissue sections mounted on the slide are nearly invisible under a light microscope so they must be stained to create contrast. ➤ Xylene removes paraffin and absolute alcohol removes the xylene, and then dip into the water. ➤ Most staining procedures in the laboratory, aside from antibody-based immunohistochemistry (IHC), use chemicals or dyes that will bind or have affinity for certain components of the cells and extracellular components. ➤ Potential contamination during the staining procedures may be much higher than having “floaters” when mounting sections from a water bath. ➤ Contamination in the staining solution is dependent on the volume of slides being stained and the time point during the day that the samples are taken as the staining baths are a potential reservoir of tissue contaminants.

The specimens are in aqueous environment to start with (water-based) and must be passed through multiple changes of dehydrating and clearing solvents (typically ethanol and xylene) before they can be placed in molten wax (which is hydrophobic and immiscible with water). The duration and step details of the “processing schedule” chosen for a particular batch of specimens will depend on the nature and size of the specimens.

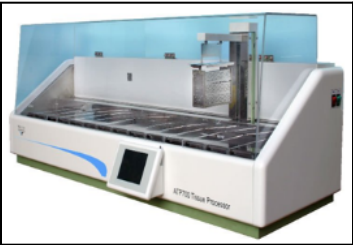


Carousel type of glass beakers is the older design of an automatic tissue processor that contains solutions and solvents which encompasses the process of dehydration and clearing. The carousel vertically agitates in each solution before moving on to the next situation in the dehydration/clearing methods. The modern processors have a chamber in which the specimens are held, and the different solutions are pumped in and out of the chamber. In general, the whole process takes around six hours and its usually set up to run overnight. Tissues that come off the tissue processor are still in the cassettes and must be manually put into the blocks by a technician who must pick the tissues out of the cassette and pour molten paraffin over them. This “embedding” process is very important, because the tissues must be aligned, or oriented, properly in the block of paraffin.

Due to the viscosity of molten paraffin wax, some form of gentle agitation is highly desirable. If the processor is to be run overnight, it should be programmed to hold on the first ethanol bath and not finish until the next morning, so the specimens do not sit in hot paraffin longer than the time indicated. If specimens are fresh, they may incubate in formalin in the first stage on the machine. It is important to not keep the tissues in hot paraffin too long or else they may become hard and brittle. Processed tissue can be stored in the cassettes at room temperature indefinitely.

AUTOMATIC TISSUE PROCESSING

Tissue Processor is a tissue processing machine that is more convenient and efficient to use in processing large volume tissues. This machine allows the specimen to be infiltrated with a sequence of different solvent finishing the molten paraffin wax.



Vacuum Infiltration is the impregnation of tissues by molten medium under reduced pressure. The procedure assists the complete and rapid impregnation of tissues with wax and reduces the time tissues are subjected to high temperatures, thereby minimizing heat induced tissue hardening, facilitating complete removal of transition solvents, and prolonging the life of wax by reducing solvent contamination.



HISTOPATHOLOGIC & CYTOLOGIC TECHNIQUES LEC

Module 6: Conventional Tissue Processing with Automation

MTHC31-24

Prof. Neil Allen Elquinto

FACTORS THAT IMPACT THE DURATION OF TISSUE PROCESSING AND EXTENT OF INFILTRATION

Factor	Description
Tissue Density and Thickness	➤ Density affects infiltration and subsequent sectioning of tissues. ➤ Thickness of tissues may influence the rate of reagent diffusion and affect processing time.
Agitation	➤ It usually increases the flow of fresh fluids in and around the tissues. ➤ Without agitation, tissues tend to settle to the bottom of the processing device or become too tightly packed, therefore reducing surface area available for fluid exchange. ➤ Fluid interchange between processing reagents and tissues is promoted by exposure of the maximum tissue surface area.
Temperature	➤ +37°C to +45°C, for a limited time can speed up fluid penetration and tissue processing procedures. ➤ However, the temperature shall be carefully monitored, high temperature can cause the tissue to shrink and to become hard and brittle, while low temperature increases the viscosity of reagents used in tissue processing.
Vacuum and Pressure	➤ Vacuum application during tissue infiltration improves processing quality and can aid in removal of trapped air from porous tissue. ➤ Vacuum can reduce infiltration time when dealing with dense and fatty tissue specimens. It can also be applied during dehydration, clearing and infiltration improves the quality of processing in tissues such as the lung which becomes de-aerated during the process. ➤ However, duration of wax infiltration is dependent upon viscosity and is not generally reduced by applying the vacuum.

- A processor log should be kept in which the number of specimens processed, processing reagent changes, temperature checks on the wax baths and the completion of the routine maintenance schedule are recorded as part of the quality assurance program.
- Gentle washing and minimal thickness of cell layers will prevent the cells from detaching during staining. Make sure that there are enough sections to make a diagnosis and ensure that the reagents have been applied evenly on the side.
- Quality of staining can be compromised by inadequate fixation and similarly by poor tissue processing. A good technician must evaluate and determine the processing of each choice for each purpose, i.e., special stains on paraffin, frozen or cell smear preparations.
- Make sure glass slides are clean and free from debris.
- In general, needle biopsies and bloody specimens should be incubated conservatively, whereas fatty specimens can be processed for longer than average.

TECHNICAL CONSIDERATIONS

- Baskets and metal cassettes should be clean and wax-free.
- Tissues should not be packed too tightly in baskets so as to impede fluid exchange.
- Processors must be free of spilled fluids and accumulated wax accumulations.
- Fluid levels must be higher than the specimen containers.
- Timing and delay mechanisms must be correctly set and checked against the appropriate processing schedule.