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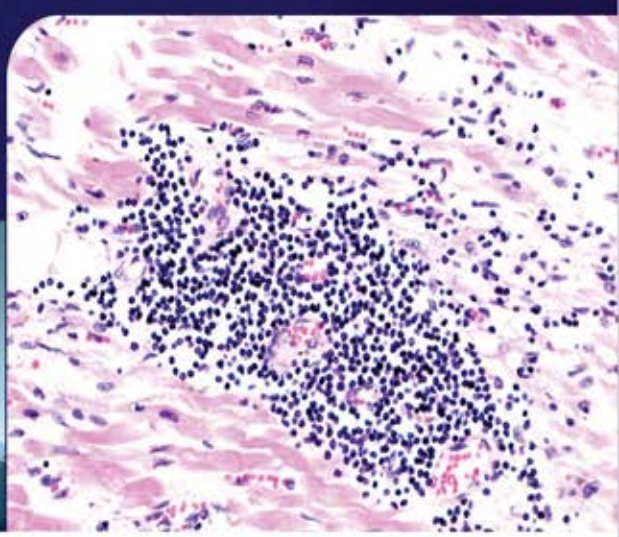
Principles & Interpretation of Laboratory Practices in SURGICAL PATHOLOGY



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Principles and Interpretation of

LABORATORY PRACTICES IN SURGICAL PATHOLOGY

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Chapter 3 Tissue Processing

Tissues must be adequately supported before sectioning for microscopic examination. Once the tissue has been fixed it must be processed into a form in which it can be cut into thin microscopic sections. Therefore, tissues are routinely taken through a series of reagents are infiltrated and embedded in a stable medium, when it becomes hard, provides the necessary support for microtomy. The treatment of tissues through these series of reagents and subsequent embedding in a medium, e.g. paraffin wax (which is similar in density to tissue thus enabling smooth sectioning) is termed as 'tissue processing'. The quality of structural tissue preservation seen in the final stained and mounted section is largely determined by the choice of fixatives, processing fluids and embedding medium. The aim of tissue processing should be minimal morphological cell distortion. When immediate sectioning is required tissues may be sectioned following a range of freezing methods as in frozen section reporting.

TISSUE SAMPLING

Tissue sampling generally follows standard protocols established by each laboratory for various categories of specimens. Tissue blocks for processing should be thin; usually 1–2 mm thick for urgent processing of specimens and 3–5 mm for routine material to be processed overnight. Specimens should not be tightly packed into processing cassettes or containers, but should have sufficient free space to facilitate fluid exchange. Small specimens and tissue fragments, e.g. endometrial biopsies, endoscopic biopsies, etc. are processed, wrapped in lens tissue and placed in fine mesh containers.

PRINCIPLES OF TISSUE PROCESSING

Tissue processing occurs due to diffusion of various substances/fluids into and out of stabilized porous tissues. The diffusion process results from the thermodynamic tendency of processing reagents to equalize concentrations inside and outside the bits of tissue, thus generally conforming to Fick's law. This law states that rate of solution diffusion through tissues is proportional to the concentration gradient (the difference between concentrations of the fluids inside and outside the tissue) as a multiple of temperature-dependent constants for specific substances.

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INITIAL PRECAUTIONS BEFORE PROCESSING

Labeling of Tissues

Specimens are generally identified by a numbering system that is not bleached by subsequent fluid and solvent treatment. Examples include:

1. A numbered card label generated by computer-printer (self-adhesive bar code label) or handwritten in soft lead pencil or waterproof ink.
2. Color-coded plastic cassettes (Tissue-Tek system), machine or manually labeled (the label should include a serial number for the specimen and that particular year, e.g. 07/2011 for the seventh specimen received in the year 2011).

Completion of Fixation

Tissues should be fixed before processing is initiated. Poorly fixed tissues are inadequately protected against the physical and chemical rigors of processing. Strategies commonly employed to ensure complete fixation of tissues include:

1. Microwave irradiation of biopsy specimens in normal saline.
2. Continuing fixation on the tissue processor with one or more changes of the routine fixative, often at elevated temperatures of 40–60°C.
3. Secondary fixation of tissues in an alcoholic fixative, which will complete fixation whilst initiating dehydration.

Postfixation Treatment

Fixation with picric acid forms water-soluble picrates, therefore, tissue blocks should be placed directly into 70% ethanol. Tissues fixed in Carnoy's fluid should be placed directly into 100% ethanol, instead of increasing graded alcohols.

STAGES OF TISSUE PROCESSING

- Dehydration
- Clearing
- Impregnation in wax (infiltration)
- Embedding
- Casting or blocking.

Each stage should be of sufficient length to ensure completeness. The tissues are transferred from reagent to reagent in tissue processing cassettes. These are metal or plastic containers with perforations. The cassettes are transported through various reagents in machines called 'tissue processors'. 'Manual processing' may also be done, where cassettes are transported manually from reagent to reagent and is done in small scale laboratories with less number of specimens. Factors affecting the rate of processing are:

1. **Specimen size:** The thicker the specimen, longer is the filtration time.
2. **Agitation:** Effective agitation reduces the overall processing time by 25–50% with improved impregnation of the tissues.

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3. **Heat:** Increases the rate of penetration and chilling decreases it.
4. **Viscosity:** The higher the viscosity of the fluid, the slower the rate of penetration.
5. **Vacuum impregnation:** Vacuum considerably reduces the impregnation time.

Dehydration

The first step in processing is dehydration. Water is present in tissues in free and bound (molecular) forms. The water needs to be removed before it is replaced by wax during impregnation and embedding. Wet-fixed tissues (in aqueous solutions) cannot be directly infiltrated with paraffin. The first stage of processing is the removal of aqueous fixatives from the tissues by various dehydrating compounds such as alcohols or a substitute. The dehydrating agents are hydrophilic and attract water molecules from the tissue or affect dehydration by repeated dilution of the aqueous tissue fluids.

Procedure

Dehydration consists of passing the tissue through series of progressively more concentrated alcohol baths. It is best accomplished by the use of graded alcohol, say 70–95% or to 100%. The transfer of tissue directly to a higher grade, i.e. 95% alcohol is risky, since it is liable to cause tissue shrinkage. Tissues are carefully transferred from one container to another at proper intervals (allowing the tissue to drain for few seconds) between each change. The volume of a dehydrating agent in each stage should be at least 10 times the volume of tissue to be dehydrated.

The concentration of the first alcohol depends on the fixative and size and type of the tissue, e.g. delicate tissue such as embryo and brain need lower concentration (50% alcohol to start with) and smaller intervals between two strengths of alcohol. Tissues immersed in alcoholic fixatives such as Carnoy's fluid may be placed directly in 100% alcohol.

Duration of dehydration should be kept to the minimum; consistent with the tissues being processed. Tissue blocks 1 mm thick should receive up to 30 minutes in each alcohol, blocks 4–5 mm thick require up to 90 minutes or longer in each change. Tissues may be held and stored indefinitely in 70% ethanol without harm. Automated programs are also available in tissue processors.

Dehydrating Agents

Alcohols: These are clear, colorless, flammable, hydrophilic liquids, miscible with water and when anhydrous, with most organic solvents. They have dehydrating as well as fixative effect (secondary fixation):

1. **Ethanol:** It is probably the most commonly used dehydrant in histology. It is supplied as 99.85% ethanol (absolute ethanol, 100%) and as special

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methylated spirits (99.85% ethanol denatured with 2% methanol). Both are satisfactory for histological purposes. There is some distortion of tissue due to shrinkage produced by immersion in ethanols for a long duration of time. Anhydrous copper sulfate acts as both a dehydrating agent and as an indicator of water content in the last bath of 100% ethanol. Anhydrous copper sulfate is layered (1–2 cm) in the bath and covered with filter paper. If water is present, the anhydrous copper sulfate turns blue.

2. **Isopropyl alcohol (isopropanol):** This is an easily available and cheaper substitute for ethanol. It is slightly slower in action, but a far superior lipid solvent than ethanol. Isopropanol shrinks and hardens tissue less than ethanol and is used to dehydrate hard, dense tissues, which can remain in the solvent for extended periods of time without harm.
3. **Butyl alcohol (butanol):** The dehydrating process is slow, requiring a longer time for immersion. It causes less hardening and shrinkage than ethanol and can be used in combination with ethanol as a dehydrating agent.

Glycol ethers: Unlike the alcohols, these reagents do not act as secondary fixatives:

1. **Dioxane (diethylene dioxide):** 1,4-diethylene dioxide causes less tissue shrinkage and hardening than ethanol. This can also be used as clearing agent because it has a unique property of being miscible with water and paraffin wax. It has a highly toxic vapor as well as high cost, hence should not be recommended for routine use.
2. **Polyethylene glycols (PEG):** These are water-miscible polymers used to dehydrate and embed substances labile to other solvents and heat of the paraffin wax method.

Other dehydrants: Include acetone, phenol, etc.:

1. **Acetone:** It is a fast and effective dehydrant. Though it may cause tissue shrinkage, it may also act as a coagulant secondary fixative. This is clear, highly volatile and easily removed by most clearing agents. Acetone is the best dehydrant for processing fatty specimens due to its fat dissolving properties. This is also a good dehydrating agent for electron microscopy. But it causes tissue shrinkage and causes brittleness, if its action is prolonged. Alcohol and acetone changes are also used in combination. Acetone is not recommended for microwave processing as it causes excessive nuclear shrinkage.
2. **Additives:** About 4% phenol added to 95% alcohol acts as both a dehydrating agent and softening agent for hard tissues such as nail, keratin masses, tendon and dense fibrous tissue.

Note: Tissues, which have been treated with a fixative containing a chromate, must be thoroughly washed in running water prior to treatment with alcohol/dioxane to remove the chromate.

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Clearing (Dealcoholization)

Clearing consists of replacing dehydrant with a substance that will be miscible with the embedding medium (paraffin) with which the tissue must be impregnated. The essential requirement of clearing agent is that it should be miscible with both dehydrating agents and impregnating agents. The clearing agents often have the same refractive index as tissues, as a result, when the anhydrous tissue is completely infiltrated with the clearing agent, it becomes translucent. This property is used to ascertain the endpoint and duration of the clearing step. The presence of opaque areas indicates incomplete dehydration. Transition solvents extract certain tissue substances such as lipids, but otherwise do not alter tissue reactivity nor behave as secondary fixatives during processing.

The boiling point of the clearing agent gives an indication of its speed of replacement by paraffin wax. Fluids with low boiling point are easily replaced. Viscosity also influences the speed of penetration.

Criteria for Choosing Suitable Clearing Agents

- Ability to rapidly remove dehydrating agents
- Easily removed by melted paraffin
- It should not produce excessive shrinkage and hardening
- It should not dissolve out aniline dyes used in staining
- Minimal tissue damage
- Least flammable
- Least toxicity
- Cost-effective.

Procedure

After dehydration, the tissue is transferred to a clearing agent. After an appropriate time interval, the tissue is transferred to a second change of clearing agent. The volume of clearing agent is optimally 30–40 times the volume of the specimen. The amount should not be less than 10 times the volume of tissue. The smaller pieces of tissue are cleared in 30 minutes to 1 hour, whereas larger tissues (> 5 mm thick) are cleared in 2–4 hours. The endpoint of clearing can be noted by transparent appearance of the tissue against light. Prolonged exposure to most clearing agents causes the tissue to become brittle and difficult to section.

Clearing Agents

Transition solvents: These are odorless flammable liquids with characteristic petroleum or aromatic odors, miscible with most organic solvents and with paraffin wax:

1. **Xylene:** The most common clearing agent in the laboratory is xylene. It is reasonably cost-effective. It clears rapidly and the tissues are rendered

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transparent, facilitating endpoint determination. Long-term immersion of tissue in xylene results in tissue distortions therefore tissues should not be left in it for more than 3 hours.

2. **Toluene:** It is better at preserving tissue structure and causes less hardening of tissues than xylene. It is a suitable clearing agent for automatic tissue processing. It is preferable for processing brain sections. However, toluene is more expensive than xylene, more toxic and a possible carcinogen, so toluene is less commonly used.
3. **Chloroform:** It is an expensive, heavy, highly volatile, slowly penetrating transition solvent. It causes less brittleness than xylene and is often used on dense tissues such as uterus and muscle. The tissues can be cleared overnight without rendering them brittle because of its tolerance. It is also good for nervous tissues, lymph nodes and embryos. But it causes severe health hazard, acts slowly and may lead to sectioning difficulties.

Esters: These are colorless flammable solvents miscible with most organic solvents and with paraffin wax. Methyl salicylate (oil of wintergreen) is safe and effective, clears tissues from 96% ethanol, hardens less and has a pleasant odor. It causes minimal tissue shrinkage and hardening and tissues can remain in it indefinitely without harm. Methyl salicylate, though, is rarely used due to its cost.

Terpenes: These are isoprene polymers found in essential oils. Terpenes clear tissues and celloidin sections from 80 to 95% alcohol, render tissues transparent and have a slow gentle non-hardening action. Most are generally regarded as safe though some have particularly strong odors, which can be overpowering, requiring good laboratory ventilation, e.g. turpentine and oils of bergamot, cedarwood, clove, lemon, *Origanum* and sandalwood.

Histo-Clear (orange oil) has been rigorously purified and stabilized. It offers the best clearing action. It is excellent for preserving fine tissue structure and can often be used in place of xylene. It also enhances the clarity and vibrancy of acidophilic stains and improves the staining of Harris hematoxylin.

Other clearing agents: Includes benzene, butyl acetate, etc.

Impregnating in Wax (Infiltration)

Impregnating is the process in which the clearing agent is replaced by paraffin or its substitute that completely fills all the tissue cavities, giving a firm consistency to the specimen and allowing easier handling and cutting of thin sections without any damage to the tissue or its cellular constituents. This is done at the melting point temperature of wax in use, i.e. 54–64°C, in case of paraffin wax. The volume of the wax should be 25–30 times the volume of the tissue.

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Criteria for Ideal Infiltrating Medium

Ideally an infiltrating and embedding medium should be:

- Soluble in processing fluids
- Suitable for sectioning and ribboning
- Capable of flattening after ribbon cutting
- Molten between 30 and 60°C
- Translucent or transparent; colorless at its melting point
- Stable
- Homogeneous
- Nontoxic
- Odorless
- Easy to handle
- Inexpensive.

Types of Wax

Paraffin wax

Paraffin wax is a polycrystalline mixture of solid hydrocarbons produced during the refining of coal and mineral oils. It is colorless or white, somewhat translucent, odorless and has a wide range of melting point, ranging from 56 to 64°C. Tissue-wax adhesion depends upon the crystal morphology of the embedding medium. Small, uniform-sized crystals provide better physical support for specimens through close packing. Crystalline morphology of paraffin wax can be altered by incorporating additives, which result in a less brittle, more homogeneous wax with good cutting characteristics. There is consequently less deformation during thin sectioning. Altering the temperature does not appreciably affect crystal size.

Paraffin wax is routinely used as an impregnating and embedding media. It is cheap, safe, immiscible with water, provides quality sectioning and is easily adaptable to a variety of uses. Paraffin wax forms a matrix that gives hardness and support to the tissues, thus preventing tissue sectioning distortion and provides easy ribbon during microtomy. Tissue blocks can be stored in paraffin wax for a long time without tissue destruction. Low melting point paraffin wax is soft and used for delicate tissues such as fetal and areolar tissues, while higher melting point paraffin wax is hard and used for hard fibrous tissues.

If tissues are processed by hand they will require a total of 4–6 hours in three changes of wax whereas with agitation, 2–4 hours in two baths will suffice. Following impregnation tissues are embedded in a wax block, which enables them to be cut into thin sections (2–8 μ thick) on a microtome. The disadvantage is that paraffin wax requires a long time of immersion to infiltrate tissues such as bones, brain, eyes, etc. Prolonged impregnation causes tissue shrinkage and hardening. All wax should be filtered routinely. If the wax crystallizes due to water content, it may be heated and stirred to remove the water.

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Modified paraffin waxes

The properties of paraffin wax are improved for histological purposes by the inclusion of substances added alone or in combination:

- Prolonged heating of paraffin wax at high temperatures or use of micro-crystalline wax improves ribboning
- To increase hardness: Add stearic acid
- To decrease the melting point: Add spermaceti or phenanthrene
- To improve adhesion between specimen and wax (alter crystalline morphology): Add 0.5% ceresin, 0.1–5% beeswax, rubber, wax or phenanthrene.

Examples of modified waxes

1. **Paraplast:** This is a mixture of highly purified paraffin wax containing plastic polymers. It has a greater degree of elasticity and provides excellent tissue infiltration and superior quality sections. It minimizes the tissue compression due to sectioning and gives wrinkle-free serial sections to be cut with ease at 4 mm thick.
2. **Paraplast plus:** This wax of highest purity contains dimethyl sulfoxide (DMSO), which gives faster penetration with a more homogeneous matrix, reduces infiltration times and facilitates thin sectioning. Tissue processing time is therefore reduced.
3. **Ester waxes:** This developed by Steedman, have low melting points (melting point 48°C). These are hard at room temperature and have good adhesive properties. Ester wax is similar to celloidin in that it can be compressed and is therefore less likely to crumble when cutting hard tissues. Like paraffin wax it gives good ribboning, thin sectioning and glass adhesion properties.
4. **Polyester wax:** This developed by Steedman, is a ribboning low melting point wax (38°C), which reduces heat-induced artifacts. It is recommended for heat-labile tissues, to minimize heat-induced hardening in difficult tissues and is an ideal medium for combined light and scanning electron microscopy.
5. **Water-soluble waxes or PEG:** These are water-soluble media used for investigation of heat- and solvent-labile lipids and proteins, and to overcome tissue shrinkage, damage and distortion inherent in the paraffin wax technique. The polyethylene glycols or Carbowax are polymers of varying length. At room temperature, PEG 600 is syrupy liquid, PEG 1000 is soft and slippery, PEG 1500 is hard, and PEG 4000 is hard and brittle. In general, they are less elastic, denser and somewhat harder than paraffin wax. For routine tissues, four changes of Carbowax with agitation (70%, 90% and two times in 100%) at 56°C is used for 30 minutes, 45 minutes and 1 hour each respectively. The specimens are then blocked in fresh Carbowax at 50°C and the blocks are prepared immediately and rapidly cooled in the refrigerator. Crystal slip is a bigger problem than in paraffin and sectioning deformation is mainly nonrecoverable.

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Advantages of PEG are:

- It eliminates dehydration and clearing, hence lipids and neutral lipids are not removed and demonstrated in thin sections
- The processing time is reduced
- Technique is good for enzyme histochemistry and immunohistochemistry
- It reduces the shrinkage and distortion.

Disadvantages of PEG are:

- They are difficult to flatten without loss of tissue and adhere poorly to slides
- As Carbowax is soluble in water, sections cannot be floated on water
- Sectioning is difficult because of tendency of the Carbowax to crumble.

Floatation fluids for these composed of:

- Diethylene glycol: 40 parts
- Distilled water: 50 parts
- Strong formaldehyde (40%): 10 parts.

Those waxes with higher (18–22) carbon atoms in their molecules have physical characteristics suitable for tissue embedding.

Vacuum Impregnation

Vacuum impregnation is the impregnation of tissues by a molten medium under reduced pressure. The procedure assists the complete and rapid impregnation of tissues with wax. It reduces the time when tissues are subjected to high temperatures, thus minimizing heat-induced tissue hardening, facilitates complete removal of transition solvents and prolongs the life of wax by reducing solvent contamination. Vacuum infiltration requires a vacuum infiltrator or embedding oven, consisting of wax baths, fluid trap and vacuum gauge, to which a vacuum of up to 760 mm Hg is applied using a water or mechanical pump.

Useful for lung tissue and tissue that contains much air. Also used for splenic tissue, which tend to become hard in routine processing. Care must be taken, while dealing with pieces of lungs, as rapid evacuation of air cause rupture of lung alveoli, which when examined microscopically may be mistaken for emphysema. Continuous supply of electricity is essential, as the apparatus cannot be opened in the middle of the procedure.

AUTOMATED TISSUE PROCESSING METHODS

Tissues are usually more rapidly processed by machine than by manual methods, although the latter can be accelerated by using microwave or ultrasonic stimulation. For routine purposes, tissues are most conveniently automatically processed through dehydration, clearing and infiltration stages by machine. There are two broad principles of automatic tissue processors, tissue-transfer and fluid-transfer types.

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Tissue-transfer Processors (Fig. 3.1 and Table 3.1)

Tissue-transfer processors are characterized by the transfer of tissues, contained within a basket, through a series of stationary reagents arranged in line or a circular carousel fashion. The rotary or carousel is the most common model of automatic tissue processor and was invented by Arendt in 1909. It is provided with 9–10 reagent and 2–3 wax positions, with a capacity for 30–110 cassettes depending upon the model. Fluid agitation is achieved by vertical oscillation or a rotary motion of the tissue basket. Processing schedules are card-notched, pin or touch pad programmed.

Tissue-transfer processors allow maximum flexibility in the choice of reagents and schedules. Metal-corrosive fixatives, a wide range of solvents, and relatively viscous nitrocellulose solutions can all be accommodated. These machines have a rapid turnaround time for day/night processing. In more recent models, the tissue basket is enclosed within an integrated fume hood to allow escape of fumes during agitation and transfer cycles thus overcoming the disadvantages of earlier styles. The main disadvantage of this type of processor is that the tissues dry, while being transferred. It is for this reason that, if electricity is interrupted, the tissue processors in routine laboratories are connected to generators.

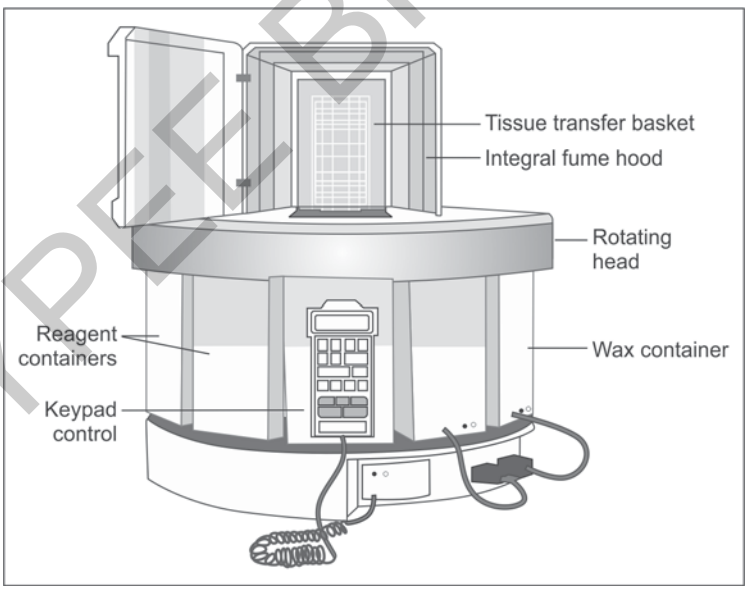

Figure 3.1: A tissue-transfer tissue processor with an integrated fume hood. Tissue cassettes are loaded into the basket on the rotating head, which transfer tissues around the series of reagent containers. Examples of this type of processors are Shandon Citadel, Technicon Ultra and Shandon Duplex.

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TABLE 3.1: Example of a processing schedule for a tissue-transfer processor

Step of processing	Duration	
	Daytime	Overnight
Fixative		120 min
Fixative		120 min
70% ethanol		60 min
90% ethanol		60 min
Absolute ethanol	30 min	60 min
Absolute ethanol	30 min	60 min
Absolute ethanol	30 min	60 min
Toluene or substitute	30 min	60 min
Toluene or substitute	30 min	60 min
Paraffin wax	30 min	90 min
Paraffin wax	30 min	90 min
Paraffin wax	30 min	90 min
Paraffin wax (under vacuum)	30 min	30 min
Embed		
Total time	4.5 h	16 h

Note: In day schedule for urgent specimens, tissues 2 mm and fixed in Carnoy's fluid. In overnight schedule for routine processing, tissue blocks 2–3 mm and single load. In weekend processing, tissues are held in fixative or preferably 70% ethanol until Sunday.

Fluid-transfer Processors (Fig. 3.2 and Table 3.2)

In fluid-transfer units, processing fluids are pumped to and from a retort in which the tissue cassettes remain stationary. There are 10–12 reagent stations with temperatures adjustable between 30 and 45°C, three to four paraffin wax stations with variable temperature settings between 48 and 68°C, and vacuum-pressure options for each station. Depending upon the model these machines can process 100–300 cassettes at one time. Agitation is achieved by tidal action. Schedules are microprocessor programmed and controlled. Vacuum pressure cycles coupled with heated reagents allow effective reductions in processing times and improved infiltration of dense tissues.

Fluid-transfer processors overcome the main drawbacks of the tissue-transfer machines. Tissue drying is prevented within the sealed retort and reagent vapors are vented through filters or retained in a closed-loop system. Processors are provided with alert systems and diagnostic programs for troubleshooting and maintenance.

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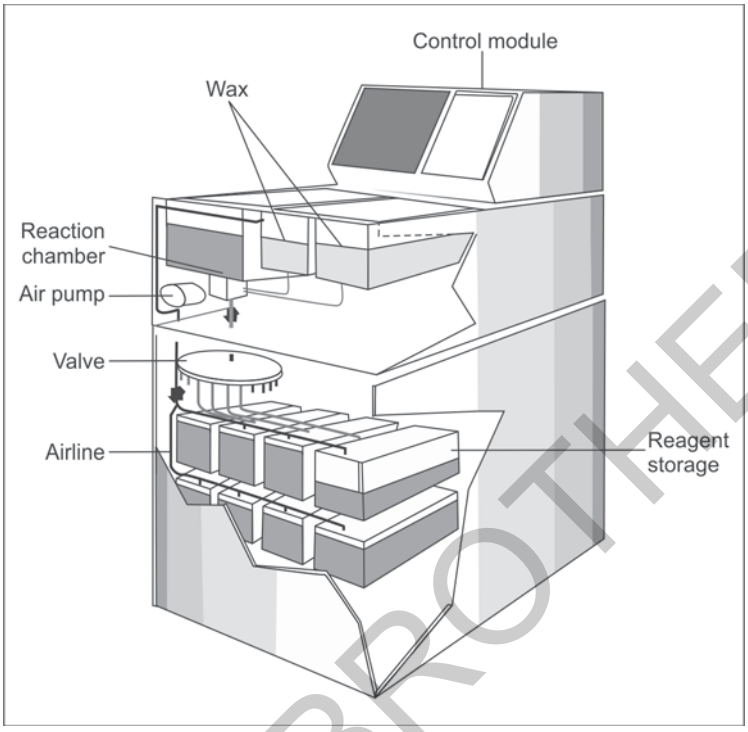

Figure 3.2: A fluid-transfer tissue processor. Tissue cassettes are loaded into the reaction chamber, which processing reagents are pumped 'to and fro' one after another. Contaminated air is not vented externally, but remains within the closed transfer system. Examples of this type are Shandon Hypercenter XP and Pathcenter or Bayers VIP.

TABLE 3.2: Example of a processing schedule for a fluid-transfer processor

Step of processing	Duration					
	Daytime			Overnight		
	Time (min)	*Temperature (°C)*	*P/V**	*Time (h)*	*Temperature (°C)*	*P/V*
Fixative				3.0	35	
Fixative				1.5	35	
70% ethanol	15		ON	1.0	40	
90% ethanol	15	40	ON	1.0	40	
Absolute ethanol	15	40	ON	0.5	45	ON
Absolute ethanol	15	40	ON	0.5	45	
Absolute ethanol	15	40	ON	0.5	45	
Absolute ethanol	15	40	ON	1.5	45	ON
Toluene or substitute	15	40	ON	0.5	50	

Contd...

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Contd...

Step of processing	Duration					
	Daytime			Overnight		
	Time (min)	*Temp-erature (°C)*	*P/V**	*Time (h)*	*Temp-erature (°C)*	*P/V*
Toluene or substitute	15	40	ON	1.5	50	ON
Paraffin wax				0.5	60	ON
Paraffin wax	15	60	ON	0.5	60	ON
Paraffin wax	15	60	ON	1.5	60	ON
Paraffin wax	15	60	ON	1.5	60	ON
Embed						
Total time (exclusive of fluid transfer time)	2.75 h			15.5		

Note: Rapid day schedule for endoscopic or needle biopsy. Overnight schedule routine for lightly fixed specimens.
*P/V, pressure vacuum option.

GENERAL CONSIDERATIONS DURING PROCESSING

1. Baskets and metal cassettes should be clean and wax-free type.
2. Tissues should not be packed too tightly in baskets so as to impede fluid exchange.
3. Processors must be free of spilt fluids and wax accumulations to reduce hazards and ensure reliability.
4. Fluid levels must be higher than the specimen containers.
5. Timing and delay mechanisms must be correctly set and checked against the appropriate processing schedule.
6. A processor log should be kept in which the number of specimens processed, processing reagent changes, temperature checks on the wax baths and completion of the routine maintenance schedule are recorded as an integral part of the laboratory quality assurance program.

TISSUE RECOVERY PROCEDURES

Procedures for recovery of tissues that have air dried because of mechanical or electrical failure of the processor or accidentally returned to fixatives are as follows. These tissues will be difficult to section and will always fall short of optimum requirements. However, the next best option is to salvage tissue to provide slides of diagnostic quality particularly where fresh tissue is not available.

Both air-dried tissues as well as tissues accidentally returned into fixative or alcohol following wax infiltration should be transferred to dehydrants

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and processed. All contaminant reagents should be discarded. Dried tissues should first be treated overnight in a solution of:

- 70% ethanol: 70 mL
- Glycerol: 30 mL
- Dithionite: 1 g.

TISSUE PROCESSING METHODS

Processing begins with dehydrating solutions and continues to completion.

Manual Tissue Processing (Table 3.3)

Manual tissue processing is usually undertaken for the following reasons:

1. Power failure or breakdown of a tissue processor.
2. A requirement for a non-standard processing schedule as for:
   a. Rapid processing of an urgent specimen.
   b. Delicate material.
   c. Very large or thick tissue blocks.
   d. Hard, dense tissues.
   e. Special diagnostic, teaching or research applications.
   f. Small scale processing requirements.

TABLE 3.3: Manual processing of 1–2 days schedule for well-fixed tissues processed using a magnetic stirrer

Step of processing	Duration	
	1–2 mm tissue thickness	3–4 mm tissue thickness
70% ethanol	20 min	1.5 h
90% ethanol	20 min	1.5 h
Absolute ethanol	20 min	1.5 h
Absolute ethanol	20 min	1.5 h
Absolute ethanol	20 min	1.5 h
Chloroform or substitute	20 min	
Chloroform or substitute	20 min	
Methyl salicylate		Overnight
Paraffin wax	20 min	1.0 h
Paraffin wax	20 min	2.0 h
Paraffin wax		1.0 h
Paraffin wax under vacuum	20 min	0.5 h
Embed		
Total time	3 h 20 min	1.5 day

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Resin Embedding

The main advantage of manual processing over automated methods lies in the flexibility of reagent selection, conditions and schedule designed to provide optimum processing for small batches of tissues. Exposure of tissues to the deleterious effects of some reagents can be carefully monitored and regulated through observation and precise timing.

Manual processing can be time-consuming and inconvenient. Care must be exercised so that tissues are left overnight in reagents that will cause minimal harm. A permanent series of solutions in wash bottles simplifies processing small single specimens. Tissues are processed in tubes and agitated on a rotor. Reagents are pipetted or decanted through a fine sieve. Multiple specimens or large blocks are economically processed in large lidded jars of processing fluids. The specimen reagent volume ratio should be at least 1:40 to 50. Agitation is provided by a magnetic stirrer.

Microwave-stimulated Processing (Fig. 3.3 and Table 3.4)

Rapid manual microwave-stimulated paraffin wax processing of small batches of tissues gives excellent results, which are comparable to tissues processed by longer automated non-microwave methods. Processing is undertaken

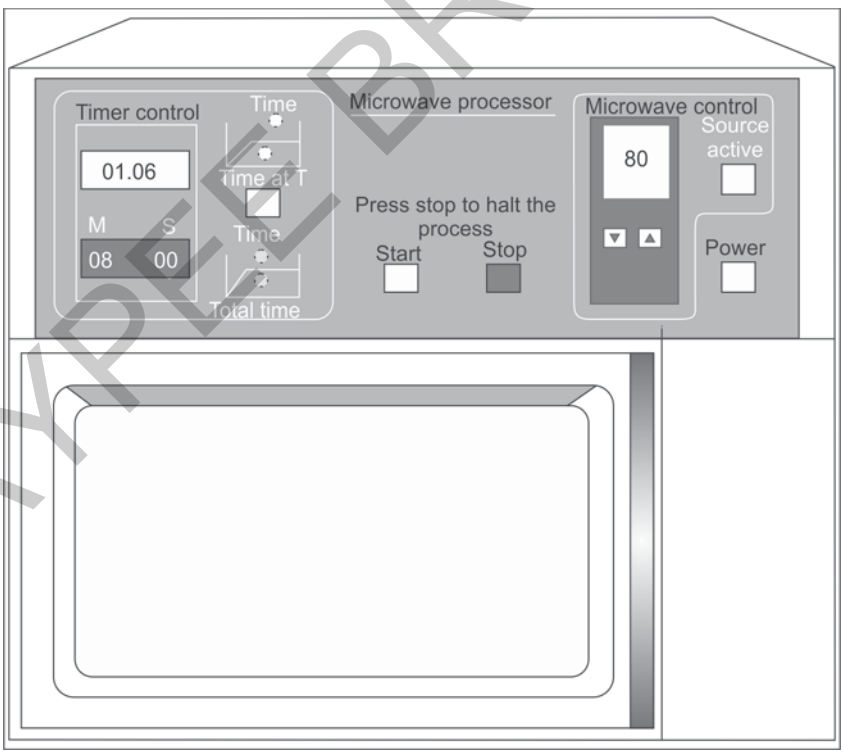

Figure 3.3: A microwave-stimulated tissue processor (T, temperature; M, minute; S, second).

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TABLE 3.4: Schedules for microwave-stimulated processing

Step of processing	Temperature (°C)	Tissue block thickness		
		< 1 mm (min)	1–2 mm (min)	2–5 mm (min)
100% ethanol	67	5	15	60
100% isopropanol	74	3	15	45
Paramat wax	67	2	15	30
	82	5	20	60
Embed				
Total time		15	65	195

in a dedicated microwave oven, which is fitted with precise temperature control and timer, and an interlocked fume extraction system to preclude accidental solvent vapor ignition. Agitation is provided by an air-nitrogen system.

Domestic microwave ovens with a temperature probe and timer accurate to seconds are suitable for tissue processing. A turntable or inbuilt radiation disperser facilitates even reagent heating. Toxic and flammable solvent vapors generated during processing cannot always be adequately vented from these ovens and present an ignition hazard if the electrical system is unprotected. Ovens should therefore be used within a fume cupboard to minimize this problem. Calibration of domestic ovens is essential for optimum results and the accuracy of the temperature probe, duration of cycle time and net power levels at various settings must be determined before the oven is used to process tissues. Tissues are placed in conventional plastic cassettes. Transparent glass or solvent-resistant plastic containers of about 200 mL capacity are ideal for processing batches of up to 14 cassettes per container.

Fixation in Microwave Oven

1. For rapid processing, tissues are fixed by microwave irradiation.
2. They can also be fixed in 95% ethanol—PEG 400 from which specimens can be transferred directly to dehydrants.
3. Formaldehyde-fixed tissues must be rinsed in running tap water for 5 minutes before microwave processing and an extra dehydration change incorporated in the schedule.

Hints for Microwave Processing

1. Tissue blocks should be as thin as possible.
2. Length and width are not as important.
3. Process blocks of similar thickness together.
4. Reagent volumes should be at least 50 times that of specimen volume.
5. The temperature probe should be placed centrally in the processing baths.
6. Use a dummy load to check whether heat generation should boil the

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reagents on minimum settings; an equal volume of reagent irradiated together with the primary load effectively halves the energy received by the primary load.

7. Preheat the paraffin wax baths in a conventional oven.
8. An increase in the number of cassettes or fluid volumes will require a concomitant increase in power and/or time to achieve the correct processing temperature.

Processing of Tissues for Electron Microscopy (Table 3.5)

The standard protocol for processing of tissues for transmissible electron microscopy (TEM) and electron microscopy involves primary fixation in an aldehyde (usually glutaraldehyde) to stabilize the proteins followed by secondary fixation in osmium tetroxide. For TEM processing, dehydration is performed by passing the specimen through increasing concentration of an organic solvent, e.g. ethanol. Commercially available absolute alcohol contains a small percentage of water, which severely restricts infiltration and polymerization of the resin used for infiltration. Hence, it is necessary to complete dehydration in anhydrous alcohol. Ethanol also requires the use of propylene oxide (1,2-epoxypropane) as a transition solvent to facilitate

TABLE 3.5: Standard processing schedule for solid tissues cut into 1 mm block (at room temperature)

Processing step	Material used/exposure	Duration
Primary fixation	2.5% glutaraldehyde in 0.1 M phosphate buffer	2–24 h
Wash	0.1 M phosphate buffer	2 × 10 min on rotator
Postfixation	1% aqueous osmium tetroxide	60–90 min
Wash	Distilled water	2 × 10 min
En bloc staining (optional)	2% aqueous uranyl acetate	20 min
Dehydration	70% alcohol 90% alcohol 95% alcohol 100% alcohol Anhydrous absolute alcohol	10 min on rotator 10 min on rotator 10 min on rotator 15 min on rotator 2 × 20 min on rotator
Transition solvent (clearing)	1,2-epoxypropane	2 × 15 minute on rotator
Infiltration	50:50 clearant of resin 25:75 clearant of resin Resin only	1 h 1 h 1–24 h (with vacuum to remove bubbles)
Embedding	Fresh resin in embedding capsules	12–24 h at 60–70°C

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resin infiltration. Propylene oxide is highly volatile, flammable and forms explosive peroxides; should be stored at room temperature in a flammable solvent facility.

Embedding

The step following dehydration is to infiltrate the tissue sample with liquid resin. In routine TEM, synthetic embedding resins are used that are capable of withstanding the vacuum in the electron microscope column and the heat generated as the electrons pass through the section. This requires gradual introduction of the resin, beginning with 50:50 mix of transition solvent (propylene oxide) and resin followed by 25:75 transition solvent-resin mix, and then finally the resin. An hour in each of the preliminary infiltration steps is usually adequate, although it is preferable to leave the samples in pure resin for 24 hours. Once infiltrated, tissue samples are placed in an appropriate mold, which is filled with resin and allowed to polymerize using heat.

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