Chapter 16 | Fixation and Tissue Processing

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Fixation

The structure of a tissue is determined by the shapes and sizes of macromolecules in and around cells. The principal macromolecules inside a cell are proteins and nucleic acids. In vertebrate animals, the macromolecules on the outside surfaces of cells and in the extracellular spaces are glycoproteins and proteoglycans, in which much carbohydrate material is covalently joined to protein molecules. Carbohydrates are hydrophilic; they hold much water in the extracellular space, by hydrogen bonding. There is also, of course, much water inside cells; water accounts for about 60% of the weight of the human body. (Guyton, Arthur C. (1976). *Textbook of Medical Physiology* (5th ed.). Philadelphia: W.B. Saunders. p. 424) In bones and teeth hydroxyapatite, a crystalline mineral containing calcium and phosphate ions, dominates the extracellular domain, together with collagen, a fibrous protein.

An essential part of all histological and cytological techniques is preservation of cells and tissues as they naturally occur. To accomplish this, tissue blocks, sections or smears are usually immersed in a fixative fluid, although in the case of smears, merely drying the preparation acts as a form of preservation. The fixatives employed prevent autolysis by inactivating lysosomal enzymes, and they stabilize the fine structure, both inside and between cells, by making macromolecules resistant to dissolution by water and other liquids. Fixatives also inhibit the growth of bacteria and molds that give rise to putrefactive changes.

In performing their protective role, fixatives denature proteins by coagulation, by forming additive compounds, or by a combination of coagulation and additive processes. A compound that adds chemically to macromolecules stabilizes structure most effectively if it is able to combine with parts of two different macromolecules, an effect known as cross-linking. The result is conformational changes in the structure of proteins and subsequent inactivation of most enzymes. Some lipids, proteins, carbohydrates and minerals are extracted by fixative liquids in which they are soluble. Fixation changes both chemical and antigenic profiles of proteins. The dilemma of fixation has always been that it introduces some artifact in order to have a protective effect. By definition, fixatives change the original chemical and physical compositions of tissues. In addition to altering the chemical nature of the cells and tissues to which they are applied, fixatives also cause physical changes to cellular and extracellular components.

Viable cells have surface membranes that are impermeable to large, hydrophilic molecules. Fixation, especially in organic liquids that dissolve or disrupt the lipids of the cell membrane, allows relatively large molecules to penetrate and escape. Furthermore, the cytoplasm becomes permeable to macromolecules, forming a proteinaceous network sufficiently porous to allow further penetration of large molecules. In this context, “large molecules” include those of paraffin wax, those of antibodies used for immunostaining, and larger dye molecules. Different fixatives result in different degrees of porosity. Coagulants such as mercuric chloride, picric acid, or zinc sulfate result in a larger pore size than do noncoagulant fixatives, such as formaldehyde, glyoxal or glutaraldehyde. Some fixatives are both coagulant and non-coagulant. For example, acetic acid coagulates nuclear chromatin but not proteins. Thus, coagulant fixatives are generally beneficial for subsequent paraffin sections. In addition to facilitating permeation of sections by antibodies, coagulant fixatives can also increase the exposure of antigenic sites, a consequence of the deformation of macromolecular shapes that constitutes coagulation. Cross-linking impedes penetration of paraffin but increases the physical strength of the tissue, especially if frozen sections are needed. Chemical addition of fixative molecules, with or without cross-linking, can modify antigenic sites and suppress immunostaining. This is a well known effect of formaldehyde, often reversible by one of many antigen retrieval procedures.

In attempts to combine the best properties of different compounds used for fixation, many mixtures have been developed. Most contain both coagulant and non-coagulant ingredients. Other substances in fixative solutions have actions such as controlling the osmotic pressure, adjusting the pH, and counteracting shrinkage or swelling caused by another ingredient. Shrinkage or swelling that occurs evenly through a specimen usually does not matter, but severe distortion results from changes in the sizes of some but not all parts of a tissue, as when tubular structures shrink within their surrounding connective tissue, or when artificial spaces are formed around cells.

Fixation before Paraffin Embedding

Most samples used for staining normal and pathological tissues are embedded in paraffin, and a number of fixatives have been formulated with this in mind. The most commonly used fixatives are discussed here. There is an abundance of specialty fixatives that will not be covered here but may be found in the references given in the bibliography.

Formaldehyde

Formalin is an article of commerce containing 40% w/v (= 40% w/w) formaldehyde (which is a gas) in water. Most of the formaldehyde is present as soluble polymers, which depolymerize on dilution. Formalin also contains about 10% methanol, which is added by the manufacturer to retard the formation of higher polymers, which eventually fall out of solution as paraformaldehyde. Old bottles of formalin, especially after storage in a cold place, often contain deposits of this white powder. Formaldehyde in solution also deteriorates by reacting with itself (Cannizzaro reaction) and changing into methanol and formic acid. Monomeric formaldehyde exists almost entirely as methylene hydrate, an addition compound formed by a reversible reaction with water. Formaldehyde itself is the compound that reacts with proteins; it is present at an extremely low concentration in a “4% formaldehyde” solution, but is produced instantly from methylene hydrate following removal from solution in the reactions of fixation. These chemical properties of formaldehyde are summarized in Figure 1.

The most commonly used fixative for histopathology is a 4% aqueous solution of formaldehyde, often called 10% formalin because it is made by tenfold dilution of formalin. For about 50 years this fixative has also included inorganic salts to maintain a near neutral pH and an osmotic pressure similar to that of mammalian extracellular fluid. The solution is called neutral buffered formalin, or NBF. It fixes not by coagulation, but by adding to the side-chains of basic amino acids, especially lysine, and to the amide nitrogen atoms of peptide linkages. Cross-linking methylene bridges are formed where two formaldehyde binding sites are close together (see Figure 1). This results in lowered permeability to macromolecules but the structures of protein molecules are not greatly altered. The small sizes of the methylene glycol and formaldehyde molecules allow rapid penetration. Consequently this fixative is suitable for large or small specimens.

Unfortunately, despite rapid penetration of tissues, the chemical reactions of formaldehyde with tissue proteins, especially the formation of methylene bridges, occur slowly. A commonly made mistake with formalin-containing fixatives is to under-fix the tissue. Small (10×10×3 mm) pieces fixed in NBF for 12-24 hours will generally show good cytoplasmic preservation and nuclear detail. Addition of formaldehyde is largely complete in 24 hours, but cross-linking reactions continue for at least two weeks. Large, soft specimens such as whole human brains require 2-6 weeks in NBF to become firm enough to cut into slices from which samples can be taken for histology. Variations in time and conditions of fixation cause the majority of problems in histochemistry.

Fixation profoundly affects histological and immunohistochemical staining, technicians, pathologists and research workers must therefore decide on the most appropriate method. Aspects to consider are temperature, size of the storage container, volume ratio, salt concentration, pH and incubation time. Formaldehyde fixation is typically performed at room temperature. Using a low and wide specimen container to allow for adequate penetration and ease of retrieval by a technician is the best choice for adequate volume ratio. In addition, 1:20 volume ratio of fluid to tissue and 3–4 mm specimen thickness are recommended for good penetration. To prevent swelling or shrinking of the cells, an isotonic solution buffered to pH 7.2–7.4 is recommended, to maintain ultrastructure and minimize cell distortion. The shorter the time elapsed between removing the sample from the body and immersing it in fixative, the better. The duration of exposure to the fixative must be optimized for each specimen type. For example, it has been noted in a CAP survey that glycogen preservation in liver can be subject to fixation artefacts. As NBF penetrates slowly through the hepatocyte membrane, the glycogen associated with the cytoplasmic protein matrix is displaced to one side of the cell. This observation is referred to as polarization and is considered part of the morphology when discerning the stain result. (Figure 2: glycogen polarization) This is a great example of how specimens that have prolonged exposure to fixatives can demonstrate extreme morphology changes. Glycogen polarization is less marked after fixation in a non-aqueous liquid, in which this polysaccharide is not soluble.

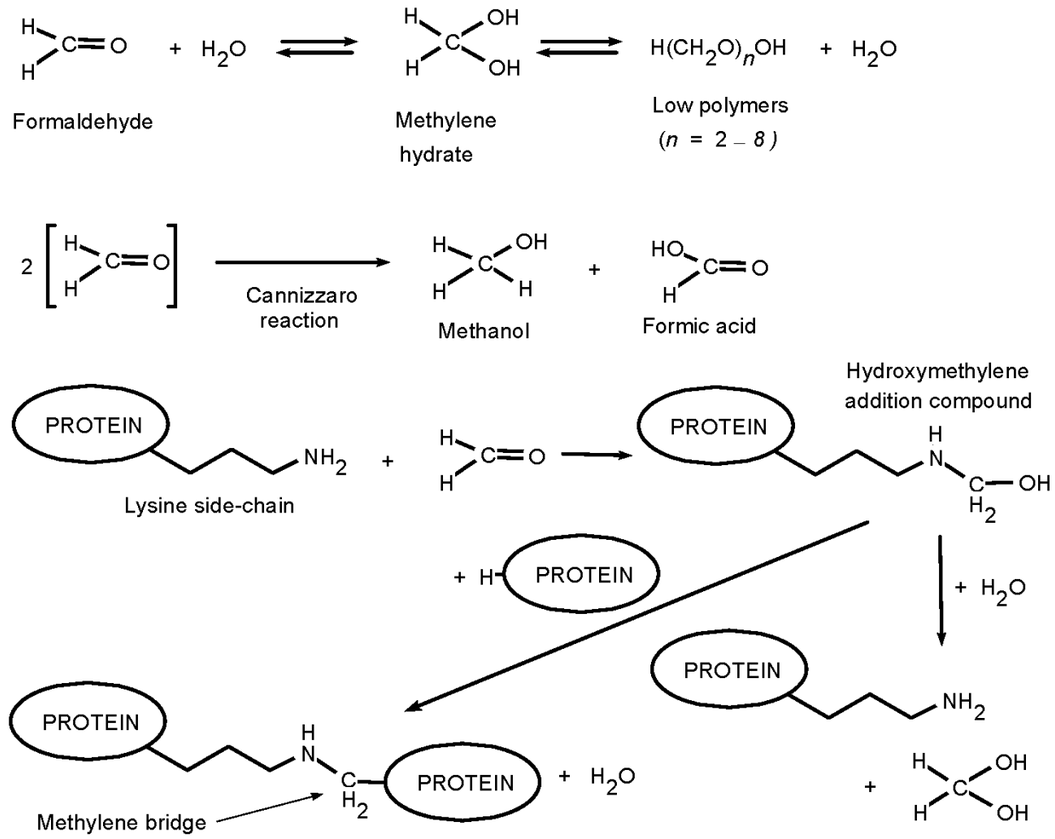

Figure 1. Some reactions of formaldehyde with water, with itself, and with proteins.

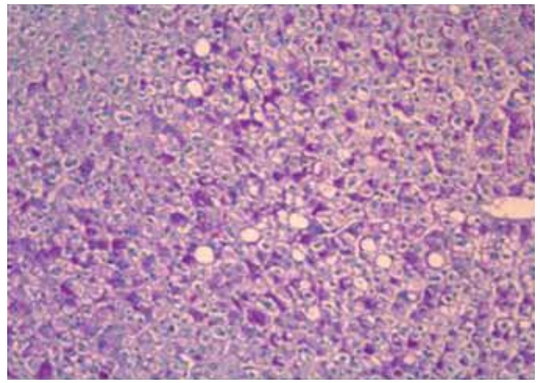

Figure 2. A paraffin section of formaldehyde-fixed liver stained by the periodic acid-Schiff method, illustrating polarization of glycogen (magenta) within the hepatocytes. Nuclei are counter-stained with hemalum (blue).

Glyoxal

Glyoxal is the simplest dialdehyde, with the formula OHC—CHO. Like formaldehyde, it readily forms hydrates and polymers. Decomposition by way of the Cannizzaro reaction occurs rapidly in neutral or alkaline solutions. Aqueous solutions must be buffered to about pH 4 to be stable, and they must also contain a small proportion of ethanol, which catalyzes the reaction of glyoxal with proteins. Addition to and cross-linking of proteins is quicker than the equivalent reactions of formaldehyde. Adequate fixation of small specimens by glyoxal is achieved in one hour.

The glyoxal-based fixatives available to laboratories are sold as pre-made solutions with trade names and undisclosed compositions. Evidently there are no published recipes for fixatives of proven efficacy that contain glyoxal as the principal ingredient. Proprietary glyoxal fixatives are reported to be useful for general histology and immunohistochemistry. The differences from formaldehyde (NBF) fixation are:

(a) Suppression of staining of arginine-rich proteins by anionic dyes (this matters if you need to stain Paneth cells, eosinophil leukocytes or the tails of spermatozoa), and

(b) Antigen retrieval is less frequently required than after fixation in NBF.

The trade secrecy associated with glyoxal-containing fixatives may preclude their use in research and in diagnostic applications where the pathologist may be interrogated about his competence and knowledge of materials used in the laboratory.

Bouin's Fluid

Pol André Bouin (1870-1962) invented several fixative mixtures in the years 1895-1900; the one most often associated with his name, first published in 1897, contains 10% formaldehyde (25% formalin), 0.9M acetic acid and 0.04M picric acid, in water. Picric acid penetrates tissues rather slowly, coagulating proteins and causing some shrinkage. It also dyes the tissue yellow. The acetic acid coagulates nuclear chromatin and opposes the shrinkage caused by picric acid. The reactions of formaldehyde have already been discussed; at the pH of Bouin's fluid (1.5-2.0) these occur more rapidly than in NBF. The complementary effects of the three ingredients of Bouin's solution work well together to maintain morphology. Specimens are usually fixed in Bouin's for 24 hours. Prolonged storage in this acidic mixture causes hydrolysis and loss of stainable DNA and RNA. Thorough washing after fixation is necessary.

Like mercuric chloride, picric acid enhances subsequent staining, especially with anionic ("acid") dyes. Paraffin sections of formaldehyde-fixed tissues are usually immersed for a few hours in a picric acid solution (Bouin's fluid is commonly used) before staining by the trichrome methods. Trichrome stains use combinations of anionic dyes with phosphotungstic or phosphomolybdic acid to impart contrasting colors to cytoplasm, collagen fibers and other components of tissues.

Mercuric Chloride

Each fixative preserves morphology differently, thus there are multiple options. For some cells and tissues NBF does not provide adequate fixation. Examples are connective tissues, some structures containing mucins, and bone marrow. Mercuric chloride ($HgCl_2$) adds to proteins and brings about rapid coagulation with resulting granularity that is too fine to be visible with light microscopy. Lillie's B5 fixative is 4% aqueous formaldehyde with 0.22M $HgCl_2$ and 0.22M acetic acid. This mixture enhances nuclear detail, which is important for identifying normal and abnormal cell types in bone marrow (hematopoietic tissue) specimens. The coagulation of nuclear chromatin is an effect of the acetic acid. The mercuric chloride ensures rapid structural stabilization and also facilitates bright staining by many of the dyes used in microtechnique. The reasons for this effect on stainability are not understood.

A dirty looking brown crystalline precipitate, probably mercurous chloride (Hg_2Cl_2) forms in all parts of tissues fixed in mixtures containing $HgCl_2$; It is called mercury pigment and must be removed, by sequential treatments with iodine and sodium thiosulfate solutions, before staining. Because it contains mercury, B5 is subject to toxic waste disposal regulations, which apply to the fixative solution and every solution thereafter that has been contaminated with mercury. Records must be kept of all B5 volumes used in the laboratory.

Zinc salts

The toxicity of mercury compounds has discouraged their use in laboratories. Of the other elements in Group 12 (IIb) of the periodic table (Zn, Cd, Hg), zinc is the least toxic. Zinc sulfate has been used for more than a century in astringent lotions and eye drops, which reduce the swelling of inflamed surfaces by coagulating extravasated plasma proteins. The first zinc-formalin fixative was probably the one published by P.A.Fish in 1895, only three years after the discovery that formaldehyde preserved tissue architecture very well. In recent decades several intelligently formulated fixatives have been developed. A few are entirely coagulant, with Zn^{2+} as the only ingredient likely to immobilize proteins. Most zinc-containing fixatives also contain formaldehyde, making them comparable to B5 and other mercury-containing solutions.

Alcoholic Fixatives

NBF is the most widely used fixative, despite not being the best one for every purpose. This is because the shortcomings of NBF are not very serious, provided that the specimens are fixed for sufficient time. Moreover, the appearances of formaldehyde-fixed tissues are familiar to pathologists, who have trained their visual systems to recognize normal and abnormal features in sections cut from routinely fixed materials.

Arguments have been made for changing the routine fixative to one of several non-aqueous mixtures, and strong cases can be made for Clarke's fluid (ethanol and acetic acid, 3:1 by volume), Carnoy's fluid (ethanol, chloroform and acetic acid, 60:30:10) and Puchtler's methacarn (Carnoy with methanol instead of ethanol). These liquids, which fix by coagulation of proteins and chromatin, contain no water. They almost completely dehydrate the tissue, so that the time for processing into paraffin wax is shorter than after NBF or other aqueous fixatives. There are also many mixtures containing an alcohol (usually ethanol), formalin, acetic acid and 10% to 70% water, often called AFA or FAA or named for their inventors, who include Tellyesniczky (around 1900), Bodian (1930s) and Davidson (1940s). In an AFA mixture the chemical reactions of formaldehyde with proteins are not retarded by buffering to a near-neutral pH. All these alcoholic fixatives contain acetic acid, which produces characteristic patterns of coagulated nuclear chromatin, facilitating the recognition of cell types. Nuclei that have been in NBF for a week or more exhibit less pronounced patterns of chromatin.

Alcohol (ethanol or methanol) alone instantly coagulates proteins but causes considerable distortion of the micro-anatomy in pieces of animal tissue. These unwanted changes are opposed by dilution of the alcohol with chloroform (immiscible with water), water, and/or acetic acid (which coagulates chromatin and opposes shrinkage, being miscible with water, alcohol and hydrocarbons. Alcohol alone (methanol is usually preferred) is suitable for fixing thin layer preparations such as blood films or cell cultures. Solid specimens taken from patients with gout are usually fixed in 95% ethanol for subsequent histochemical detection of sodium urate crystals, which can be dissolved out of the tissue by water.

Decalcification

Following fixation, there are techniques available to the histotechnician to improve microtomy and staining quality. Each technique must be evaluated for each assay to preserve morphology and provide the best stain possible (Table 1). The first method, going in chronological order of histology, is decalcification of specimens that may be difficult to cut on a microtome because of calcium carbonate or phosphate deposits. Decalcification can be achieved either by acids or by chelating agents. First, make sure the tissue has been adequately fixed and rinsed well to prevent any undesired reaction with the decalcifying agent. Dilute mineral acids (hydrochloric or nitric) or formic acid can be used effectively if the end point of decalcification is monitored carefully. Nuclear and cytoplasmic detail are compromised if specimens are exposed for too long to acidic decalcifying agents, which can extract RNA and remove the purine and pyrimidine bases from DNA. It is also imperative to wash the acid out of the tissue. If preservation of nuclear DNA is important, or if histochemical methods for nucleic acids or enzyme activities are intended, a chelating agent is preferred to an acid. Usually the disodium salt of EDTA is used, with the pH adjusted to a level between 7 and 8. Decalcification by EDTA much longer than decalcification by acids – weeks rather than days.

Table 1. Stain optimization.

Stain Technique	Thickness of Sections	Recommended Fixative	Fixatives to Avoid
AFB	3–4 µm FFPE smear	NBF 95%	
Bielschowsky	3–4 µm FFPE frozen	NBF	
Bodian	3–4 µm	NBF	
Congo Red	8–10 µm FFPE frozen	NBF	
PAS	3–4 µm FFPE frozen 10 µm	NBF	
Jones	2 µm FFPE	NBF	
GMS	3–4 µm FFPE smear	NBF 95%	
Urate Crystals	3–4 µm	alcohol	aqueous, NBF
Iron	3–4 µm smear	NBF air dry	
Reticulin	3–4 µm	NBF	
Snook's			
Gram	3–4 µm smear	NBF 95%	
Warthin-Starry	3–4 µm	NBF (critical)	
Helicobacter	3–4 µm	NBF	
Masson's Trichrome	3–4 µm	NBF, Bouin's	
Colloidal Iron	3–4 µm	NBF	chromate
Copper	3–4 µm	NBF, B5 okay	Bouin's, decal
Rhodanine			
TRAP	smear	citrate/acetone	
Sudan Black	3–4 µm frozen 10 µm	NBF	Bouin's
Leder	3–4 µm	NBF, B5	decal
Mast Cell	smear	CAF*	
MPO +/- Fluoride	smear	alcoholic formalin	
Alcian Blue	3–4 µm	NBF	
Mucicarmine	3–4 um	NBF	
Feulgen	3–4 µm	NBF	Bouin's
Gomori's Trichrome	3–4 µm	NBF	
Elastic Van Gieson	3–4 µm	NBF	
Oil Red O	3–4 µm	NBF	
Fontana Masson	3–4 µm	NBF	
Calcium	3–4 µm	NBF, alcohol	
Bile	3–4 µm	NBF	
PTAH	3–4 µm	B5	
Mallory's			
Luxol Fast Blue	3–4 µm	NBF	

*CAF = citrate + acetone + formalin

Frozen Sections

For histochemistry, cryostat sections give much faster results than paraffin sections. Additionally, fixative can be used with cryostat sections, allowing the histochemist to select a different and optimal fixative for each stain, all from the same sample. The morphological detail and resolution of frozen sections is usually considerably inferior to tissue that has been embedded in paraffin. In histopathology, frozen sections are commonly cut from muscle and nerve biopsies and from surgically removed tumors. Muscle and nerve biopsies are subdivided into specimens for formalin fixation and paraffin embedding, unfixed snap-frozen for cryostat sections, fixation and resin embedding for electron microscopy (EM) and, in some rare cases, biochemical immunoblotting studies. Multiple fixation processes are required because multiple techniques are to be used. The portion of a specimen portion intended for frozen sectioning should be transported on top of wet ice, on saline-dampened gauze, and rapidly frozen within two hours. Do not allow the tissue to freeze slowly or to soak up excess saline, as these will cause artifacts that can be seen microscopically and interfere with diagnostic interpretation.

It has been suggested that talc powder can alleviate the moisture absorbance of the muscle tissue. This procedure should be evaluated and quality tested before introducing it into a laboratory. Upon receipt in the histology lab, orient in OCT (optimal cutting temperature) compound and snap-freeze in liquid nitrogen/isopentane for optimal results. For complete instruction and illustration, refer to pg 312-314 in Carson & Hladik's textbook. Orientation, size, and expedient flash freezing are critical to obtaining undamaged sections of unfixed muscle fibers. The EM portion of a biopsy will be fixed in a buffered solution of glutaraldehyde and postfixed in osmium tetoxide, usually by a specialist in electron microscopy. In some muscular degenerative disorders, biochemical techniques may also be required.

Smears

Histotechnicans sometimes perform special stains on cytology smears, blood films and cytopreps from other departments within the laboratory. Increasingly, the commonly received cytoprep is that of the "thin prep." These smears are wet-fixed in 95% ethanol immediately after preparation to preserve the fine structure of the chromatin and help in the evaluation of nuclear changes. The May-Grünwald or the Giemsa stain is routinely evaluated, and the more complicated Papanicolaou method is also widely used, especially on samples taken from the vagina and cervix. Air drying is avoided with smears for cytological detection of neoplasia because it changes the appearances of the cells. Slides bearing blood or bone marrow smears, on the other hand, are usually air-dried. Marrow smears are stained in parallel to sections of the bone marrow core biopsy.

Specimen Processing

Staining quality can be depreciated by inadequate fixation and similarly by poor tissue processing. A good technician must evaluate and determine the processing of choice for each purpose, be it special stains on paraffin, frozen or cell smear preparations.

Paraffin processing has evolved and stabilized in the modern histology lab with the use of vacuum infiltration. It remains fundamentally important to remember the basics when processing and troubleshooting. The specimen processing methods presented in this chapter should be considered a brief introduction and do not include all the available procedures.

Tissue Processing

In order to prepare a tissue for embedding, it must be infiltrated with paraffin. Because water and paraffin are not miscible, the specimens must be gradually dehydrated to achieve replacement of water with alcohol before the clearing agent is introduced. The size and penetrability of the tissue dictate how quickly this will occur. Once successfully dehydrated, a clearing agent that is miscible with alcohol and paraffin (i.e. xylene or substitute) is infiltrated through the tissue. Finally, the paraffin is introduced and completes the tissue for embedding. A vacuum automated system improves the efficiency of wax infiltration by speeding up the removal of the clearing agent.

Keep in mind that individual laboratories must optimize to their specimen types. In general, needle biopsies and bloody specimens should be incubated conservatively, whereas fatty specimens can be processed for longer than average.

In recent years efforts have been made to streamline processing time by using microwave heating techniques. The benefits and risks associated with the technique are being revealed as more laboratories employ the technology and publish their findings. For a list of easily available literature, review the bibliography section. At this time, the concept will be presented with supporting examples.

It has been reported that microwave processing can be achieved ~60% faster than conventional processing time. See Table 2 for a comparison of the two techniques, each using 1-3mm thick biopsy samples.

One critical aspect of using microwave techniques is ensuring the samples have been adequately fixed. Each laboratory should evaluate its fixation control methods to optimize the use of microwave processing. Although the decrease in processing time increases the specimen workflow, there has been some resistance to the implementation of microwave processing technology, partly for staffing reasons. Traditionally histopathology staffs are available in the morning hours when conventional overnight processing yields its highest volumes, whereas microwave processing may keep staff later in the day. Microwave-accelerated processing is as effective as slower traditional processing, and sections stain identically with several methods: Periodic Acid-Schiff's, Van Gieson, Congo red, Masson's trichrome, alcian blue, Mayer's mucicarmine, and silver methods for reticulum. Because staining methods can vary from lab to lab, it is recommended that individual labs validate microwave methods within their environments.

Whether the lab has time initially to validate the technique and train the staff accordingly, long term considerations are prevalent. Converting to the safer chemicals (ie. less fumes, non-regulated disposal) may include rotating smaller quantities more often, causing a net increase in chemical consumption. The safety benefits of removing undesired regulated waste in addition to calculating net volumes may offer immediate cost savings. Each lab must weigh its benefits to fit its needs.

Table 2.

Process	Solution	Time	Retort
a) Conventional, room temperature processing schedule, 3mm biopsy. 12 hrs.			
Fixation	Formalin, 10%	120 min	---
Fixation	Alcoholic formalin	60 min	---
Fixation	Alcoholic formalin	60 min	---
Dehydrate	Alcohol, 95%	60 min	vacuum
Dehydrate	Alcohol, 95%	45 min	---
Dehydrate	Alcohol, absolute	45 min	vacuum
Dehydrate	Alcohol, absolute	60 min	---
Clearing agent	Xylene	60 min	---
Clearing agent	Xylene	60 min	vacuum
Infiltrate	Paraffin	30 min	---
Infiltrate	Paraffin	60 min	---
Infiltrate	Paraffin	90 min	vacuum

Table 2.

Process	Solution	Time	Retort
b) Conventional processing schedule, 1mm biopsy. 2.5 hrs.			
Dehydrate	Alcohol, 65%	15 min	
Dehydrate	Alcohol, 95%	15 min	
Dehydrate	Alcohol, 95%	15 min	
Dehydrate	Alcohol, absolute	15 min	
Dehydrate	Alcohol, absolute	15 min	
Clearing agent	Xylene	15 min	
Clearing agent	Xylene	15 min	
Infiltrate	Paraffin	15 min	
Infiltrate	Paraffin	15 min	
Infiltrate	Paraffin	15 min	
c) Microwave processing schedule, 1mm biopsy. ~45 min.			
Dehydrate	Ethyl alcohol, 100%	---	fill
DRAIN			
Dehydrate	Ethyl alcohol, 100%	5 min	67 °C, microwave
DRAIN			
Dehydrate	Isopropyl alcohol, 100%	3 min	74 °C microwave
DRAIN			
Infiltrate	Liquid paraffin, 60 °C		fill
DRAIN*			
Infiltrate	Liquid paraffin, 60 °C	2 min	65 °C microwave, agitate
	---	5 min	85 °C microwave
DRAIN*			

*Drain to 80 °C container

Embedding and Microtomy

Once the tissue has been processed it is ready to be orientated into a paraffin block and subsequently sectioned. Orientation during embedding is crucial for the representation of proper morphology. Structures in skin, small gastrointestinal biopsies, and vas deferens are among those for which orientation is especially critical. Good microtomy techniques will minimize artifacts that lead to difficult diagnostic interpretation of special stains. One of the most directly correlated factors is the thickness in which a specimen is cut. Routine H&E stained specimens are cut 3–4 µm, but some morphology is best represented otherwise. For example, amyloid deposits are better represented at 8–12 µm, whereas kidney biopsies should be cut at 2 µm for optimal viewing of the structures of glomeruli. Techniques often used to aid in microtomy are water bath adhesive and positively charged slides. However, in some silver impregnation stains, the silver ions are attracted to the coating and produce an overall background to the slide. To avoid this artifact (Figure 3), use clean slides without a coating.

After sectioning, the tissue slide is drained and may be gently heated to evaporate the layer of water between the sections and the glass. When all the water is gone, it is permissible to heat the slide enough to melt the wax, a procedure that may improve adhesion. For optimal melting of paraffin consult the melting temperature and “plastic point” on the manufacturing product insert. The plastic point is usually a few degrees lower than the melting point and represents the lowest temperature at which permanent deformation can occur without fracture.

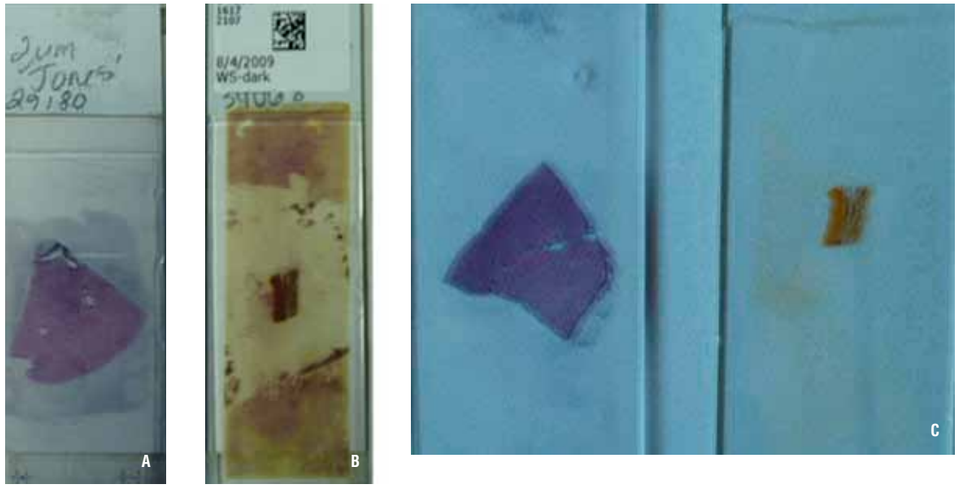

Figure 3. **A.** Jones’ silver method, kidney, 2µm adhesive residue stained by a silver method. **B.** Warthin Starry 4µm paraffin residue ghost (not stained by reduced silver). **C.** Jones’ and Warthin Starry slides demonstrating no mirroring artifact.

Technical Considerations

There are some technical tips that can apply to all preparative procedures in microtechnique. Make sure glass slides are clean and free from debris. Gentle washing and minimal thickness of cell layers will prevent the cells from detaching during staining. Staining interpretation depends on adequate chemical spread and allocation. Make sure that there are enough sections to make a diagnosis, and that ensure that the reagents have been applied evenly to the slides. If counterstaining is required, be sure to not over-incubate.

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