2 Laboratory Techniques

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General Comments

The modern surgical pathology laboratory is equipped to perform a staggering number of routine and special diagnostic procedures. Most are carried out by the laboratory technologist; however, gross room personnel should be familiar with the basic concepts and initial steps of these procedures. Failure to handle tissue appropriately may preclude the performance of needed diagnostic studies and ultimately delay or even prevent the establishment of a diagnosis. This chapter provides a basic introduction to the more common techniques employed in tissue fixation, staining, decalcification, and intraoperative consultation.

Fixation

Adequate fixation by an appropriate fixative is central to any histologic preparation. Tissue that is inadequately or inappropriately fixed will lead to difficulties in microtomy, staining, and performing ancillary tests. These problems may not be correctable at a later stage.

Unfortunately, there is no ''all-purpose'' fixative. No single fixative is good for all specimens. It is therefore essential that surgical pathology personnel be familiar with a variety of fixatives and their uses. Although the exact mechanism of action of many fixatives is unknown, fixatives can broadly be classified into four groups based on their mechanism of action. The aldehydes, such as formaldehyde and glutaraldehyde, act by cross-linking proteins, particularly lysine residues. Oxidizing agents, such as osmium tetroxide, potassium permanganate, and potassium dichromate, also probably cross-link proteins, although their precise mechanism of action is unknown. Acetic acid, methyl alcohol, and ethyl alcohol are all protein-denaturing agents. The fourth and final group of fixatives acts by forming insoluble metallic precipitates, and these agents include mercuric chloride and picric acid. The choice of the appropriate fixative is based on the type of tissue being fixed and on projected needs for ancillary tests such as special stains, immunohistochemistry, *in situ* hybridization, and electron microscopy. Table 2-1 lists some common fixatives, their basic uses, and their advantages and disadvantages.

Ten percent neutral buffered formalin (4% formaldehyde) is the standard fixative used in most laboratories. Formalin tends to remove water-soluble substances such as glycogen, and it is therefore generally not suitable for the fixation of tissues for electron microscopy. Ten percent neutral buffered formalin penetrates and fixes tissues at a rate of approximately 2 to 3 mm/24 h at room temperature.

Glutaraldehyde, a common fixative for electron microscopy, is one of the slowest penetrating fixatives. Tissue for electron microscopy should be cut into 1-mm cubes and immediately placed in refrigerated glutaraldehyde. Glutaraldehyde (4%) must be kept refrigerated before use.

Ethyl alcohol (70% to 100%) is seldom used as a primary fixative. It may be useful in fixing tissue for preserving glycogen and for some histochemical studies, but it has several disadvantages. Ethyl alcohol penetrates tissues very slowly, and because it denatures proteins by abstracting water from the tissue, it can cause excessive hardening, tissue shrinkage, and cell distortion. Alcohol can also dissolve fats and should not

TABLE 2-1. Common fixatives.

Fixatives and their major components	Tissue	Special stains (+ = stain can be used with fixative) (− = stain should not be used with fixative)	Advantages (+) and disadvantages (−)
10% Neutral buffered formalin (4% Formaldehyde, 7.2 pH phosphate buffer, methanol)	All	+ Warthin-Starry (spirochetes) + Oil red O (fat) + Grimelius (neuroendocrine granules)	+ Routine fixative + Preservation, general staining + Immunohistochemistry + Molecular analyses + Long-term storage
2% Glutaraldehyde (50% Glutaraldehyde, 0.2 M cacodylate buffer)	All	−PAS (false positivity)	+ Electron microscopy + Preservation of collagen − Routine fixative − Slow penetration − Must be refrigerated
B5 (Mercuric chloride, sodium acetate, 37% formalin)	Lymph node Spleen Bone marrow		+ Cytoplasmic, nuclear staining − Routine fixative − Must be prepared fresh − Requires iodine treatment to remove mercury before routine staining − Timed exposure needed because overfixation causes hardening
Zenker's (Mercuric chloride, potassium dichromate, sodium sulfate, glacial acetic acid)	All	+ Sheehan (chromaffin) + Mallory's PTAH (collagen and muscle) + Viral inclusions (Negri bodies) + Feulgen (DNA) + Trichromes (collagen and muscle) + Verhoeff-van Gieson (elastic fibers)	+ Routine fixative + Preserves mitochondria − Must be washed overnight to remove excess chromate − Requires iodine treatment to remove mercury before routine staining − Must be prepared fresh − Molecular analyses − No metal instruments − Immunohistochemistry
Zenker's formol (Helly's fluid) (Mercuric chloride, potassium dichromate, sodium sulfate, 37% formaldehyde)	Bone marrow Spleen All blood-containing organs		+ Routine fixative + Preserves mitochondria + Preserves red blood cells − Must be washed overnight to remove chromate − Requires iodine treatment to remove mercury before routine staining − Molecular analyses − Immunohistochemistry
Bouin's (Picric acid, formalin, glacial acetic acid)	Testicular biopsies	+ Masson trichrome (collagen and muscle)	+ Routine fixative − Lyses red blood cells − Removes ferric iron − Dissolves some proteins − Molecular analyses − Immunohistochemistry − Carbohydrates

TABLE 2-1. *Continued.*

Fixatives and their major components	Tissue	Special stains (+ = stain can be used with fixative) (− = stain should not be used with fixative)	Advantages (+) and disadvantages (−)
Ethyl alcohol	All	+ Congo red (amyloid) + Von Kossa's (calcium) + Weigert's stain for fibrin + Mallory's stain for iron + Gomori's methenamine silver stain for urate crystals − Ziehl-Neelsen (AFB)	+ Enzyme histochemistry + Molecular analyses + Impression smears + Blood smears + Preserves glycogen + Preserves crystals: uric acid, sodium urate − Causes excessive hardening − Routine fixative − Dissolves lipids
Acetone 0–4°C	All	− Ziehl-Neelsen (AFB)	+ Enzyme histochemistry − Routine fixative − For best results, must be refrigerated − Dissolves lipids
Carnoy's (Glacial acetic acid, absolute ethanol, chloroform)	All	+ Methyl green pyronin (DNA and RNA) + Congo red (amyloid) + Giemsa (mast cells) − Ziehl-Neelsen (AFB)	+ Cytologic fixative + Rapid penetration + Nuclear detail + Fixes RNA + Preserves glycogen − Dissolves cytoplasmic elements − Hemolyzes red blood cells

be used when lipid studies or stains for myelin are being considered. Carnoy's is a fixative that combines ethanol, chloroform, and glacial acetic acid. It quickly fixes tissues and it is a good fixative for glycogen, plasma cells, and nucleic acids. Because of its quick action, some laboratories use Carnoy's to fix biopsies that require urgent processing.

The mercury-based fixatives (e.g., B5) provide excellent nuclear detail and are useful in evaluating lymphomas. Mercury-based fixatives precipitate proteins without firmly binding to them. These fixatives generally must be prepared fresh; once fixed, the tissues require special processing in the histology laboratory (iodine treatment to remove the mercury). Overfixation with B5 can cause excessive hardening of the tissue.

Bouin's, a picric acid-based fixative, is the fixative of choice for testicular biopsies. Picric acid reacts with basic proteins and forms crystalline picrates with amino acids. Therefore, tissues fixed with picric acid-based fixatives retain little affinity for basic dyes, and the picric acid must be recovered from the tissue before staining. Picric acid penetrates tissues well and fixes them rapidly, but it also causes cells to shrink. Picric acid causes DNA methylation; hence, many polymerase chain reaction (PCR)-based molecular diagnostic tests cannot be performed on tissues fixed with picric acid.

An appropriate fixation technique is just as important as choosing the correct fixative. Appropriate fixation requires adequate tissue exposure and a duration of fixation sufficient to allow full penetration of the fixative. For most tissues, a volume of fresh fixative 15 times the volume of tissue is needed to fix the tissue adequately within 12 to 18 hours. The rate of fixation varies depending on the type of fixative, the type of tissue, and the thickness of the tissue sections. Adipose tissue (due to its hydrophobic nature) and fibrous tissue (due to its density) may require longer periods of fixation when hydrophilic fixatives are employed.

There can be no more important tenet of fixation than to do it early. The process of autolysis begins immediately, and even the best fixative can only arrest, not reverse, this process. Small

amounts of tissue may arrive in fixative or saline, whereas larger tissues usually arrive fresh. Large specimens generally do not fix well unless first prepared. Even then, specimens often require a limited dissection to maximize the surface area exposed to the fixative, thereby ensuring adequate fixation. Tissue with a hollow viscous or lumina should be opened and solid tissue partially serially sectioned at 5- to 10-mm intervals. To maintain proper orientation, these partially sectioned tissues can be pinned onto a wax block and floated in a fixation tank. Paper towels can be inserted between the sections. The towels act as a wick, drawing more fixative to the sections, thereby facilitating rapid fixation. In general, tissue submitted for processing should never exceed a thickness of 4 mm, and tissues comprised of adipose or dense fibrous tissue should be no more than 3 mm. Optimally, you should routinely aim to submit your tissue sections as 2 mm slices. There should be at least a 3-mm space between the cassette and tissue on all sides. Cramming oversized tissue to make it fit into a cassette often results in inferior slide preparation, time consuming reprocessing, and ultimately a delay in diagnosis.

Special Stains

A variety of special stains are employed in the surgical pathology laboratory. Most of these are performed by specially trained laboratory personnel, and a detailed description of all of these stains is beyond the scope of this manual. The surgical pathologist and pathologist's assistant, however, should be able to perform a few basic stains: hematoxylin and eosin (H&E), oil red O (ORO), periodic acid-Schiff (PAS), Gram Weigert's (GW), Ziehl-Neelsen, and Papanicolaou (PAP) stains.

While the details of each stain vary from laboratory to laboratory, examples of ''cookbook-style'' instructions for the performance of each of these six stains on frozen tissue sections follow.

Stain: **Hematoxylin and Eosin (H&E)**
Approximate time: 3 minutes
Technique: 4 to 6 μm frozen section, touch prep, smear, crush prep
Any slides
Fixative: Absolute alcohol
Procedure:
1. Absolute alcohol, 60 seconds
2. Rinse in tap water (one change); dip until clear
3. Hematoxylin (Harris), 60 seconds
4. Rinse in tap water (three changes); dip until clear
5. Blue in Scott's water, 15 seconds
6. Rinse in tap water (two changes); dip until clear
7. Eosin-phloxine, 5 seconds
8. 50% Alcohol, five dips
9. 70% Alcohol, five dips
10. 95% Alcohol, five dips
11. Absolute alcohol, five dips (two changes)
12. Xylene (two changes); dip until clear
13. Mount and coverslip with resinous media

Results: Nuclei: blue to purple
Cytoplasm: pink to red

Stain: **Oil Red O (ORO)**
Approximate time: 10 minutes
Technique: 6 to 8 μm frozen section, touch prep, smear, crush prep
Poly-L-lysine or sialinated slides
Fixative: 10% Neutral buffered formalin
Procedure:
1. 10% Neutral buffered formalin, 60 seconds
2. Wash in tap water and blot dry
3. Oil red O, 5 minutes
4. Wash in tap water
5. Hematoxylin (Harris), 60 seconds
6. Rinse in tap water (two changes)
7. Blue in Scott's water, 15 seconds
8. Rinse in tap water (two changes)
9. Mount and coverslip with *aqueous* media

Results: Fat: orange to bright red
Nuclei: blue
Cytoplasm: transparent

Stain: **Periodic Acid-Schiff (PAS)**
Approximate time: 35 minutes
Technique: 4 to 6 μm frozen section,
Poly-L-lysine or sialinated slides

Fixative: Absolute alcohol

Procedure
1. Absolute alcohol, 60 seconds
2. Rinse in distilled water (one change)
3. 0.5% Periodic acid solution, 5 minutes
4. Rinse in distilled water (three changes)
5. Schiff reagent, 15 minutes
6. Wash in running tap water, 2 to 5 minutes or until pink color develops
7. Hematoxylin (Harris), 60 seconds
8. Wash in tap water, 2 minutes
9. 50% Alcohol, five dips
10. 70% Alcohol, five dips
11. 95% Alcohol, five dips (two changes)
12. Absolute alcohol, five dips (two changes)
13. Xylene (two changes); dip until clear
14. Mount and coverslip with resinous media

Results: Fungi: red to rose
Glycoproteins, mucopolysaccharides: red to rose
Nuclei: blue
Cytoplasm: light pink

Stain: **Gram Weigert's (GW)**
Approximate time: 10 minutes

Technique: 4 to 6 μm frozen section, touch prep, smear, crush prep
Poly-L-lysine or sialinated slides

Fixative: Absolute alcohol

Procedure:
1. Absolute alcohol, 60 seconds
2. Eosin, 60 seconds
3. Rinse in tap water (one change)
4. Sterling's gentian violet, 60 seconds
5. Rinse in tap water (one change)
6. Gram's iodine, 2 minutes
7. Blot slides
8. Differentiate with aniline/xylene mixture (two to three changes) until the black color is lost.
9. Xylene (two changes); dip until clear
10. Mount and coverslip with resinous media

Results: Gram-positive bacteria and *Pneumocystis carinii:* purple to blue
Nuclei: blue
Cytoplasm: light pink

Stain: **Modified Ziehl-Neelsen** (Acid Fast)
Approximate time: 60 minutes

Technique: 4 to 6 μm frozen section, Poly-L-lysine or sialinated slides

Fixative: 10% Neutral buffered formalin

Procedure:
1. Absolute alcohol, 60 seconds
2. Rinse in tap water
3. Carbol-fuchsin, 45 minutes
4. Wash in tap water
5. 1% Acid alcohol (one to two quick dips)
6. Wash in tap water
7. Methylene blue, 10 to 20 seconds
8. 50% Alcohol, five dips
9. 70% Alcohol, five dips
10. 90% Alcohol, five dips (two changes)
11. Absolute alcohol, five dips (two changes)
12. Xylene (two changes); dip until clear
13. Mount and cover slip with resinous media

Results: Acid-fast bacilli: bright red
Background: light blue

Stain: **Papanicolaou (PAP)**
Approximate time: 60 minutes

Technique: Touch prep, crush prep, smear, fine needle aspiration
Poly-L-lysine or sialinated slides

Fixative: 95% Alcohol

Procedure:
1. 95% Alcohol, 15 minutes
2. 75% Alcohol, five dips
3. 50% Alcohol, five dips
4. Distilled water, five dips
5. Hematoxylin, two to three minutes

6. Wash in tap water
7. 0.25% HCl, one to two quick dips
8. Wash in tap water
9. 30% Alcohol, five dips
10. 75% Alcohol, five dips
11. 95% Alcohol, five dips
12. Orange G, 2 minutes
13. 95% Alcohol, five dips (three changes)
14. Eosin-azure 50, 2 minutes
15. 95% Alcohol, five dips (two changes)
16. Xylene, five dips (three changes)
17. Mount and cover with resinous material

Results: Nuclei: blue
Cytoplasm: pink, gray, to green

Immunohistochemical Stains

Immunohistochemical studies employ an unlabeled antibody to a specific tissue antigen followed by treatment in one or more steps with an enzyme-labeled antibody. These studies are extremely versatile and can be used to detect an ever-expanding number of antigens. Depending on the specific antigens, they can be performed on fresh-frozen or formalin-fixed, paraffin-embedded tissues. The effectiveness of immunohistochemistry depends on the integrity, stability, and availability of the target antigen. Three steps can improve the results of your immunohistochemical staining. First, sample a viable and representative area of the process to be studied. Immunohistochemical stains of necrotic material are practically worthless. Second, choose the appropriate method of stabilizing the tissue. While formalin is generally a good fixative for most immunohistochemical stains, some stains work best in other fixatives, and other stains work only on fresh-frozen tissue. Finally, do not overfix the tissue. Overfixation may result in the loss of antigenicity. In general, tissue fixation for longer than 24 hours compromises immunohistochemical analysis.

Decalcification

A wide variety of calcified specimens are received in the surgical pathology laboratory. While some of these should be cut with specialized, expensive equipment, the vast majority of calcified specimens can be handled by a routine histology laboratory if they are appropriately decalcified. Decalcification is the process whereby calcium salts are removed from bone and other calcified tissues. Three general methods are employed to decalcify tissues. These include acid hydrolysis, organic chelation, and electrolysis (see Table 2-2). The important points to remember about decalcifying specimens follow:

1. The tissue must be fixed before decalcification. In most cases, fixing a specimen for at least 24 hours in 10% neutral buffered formalin is adequate. If you use a different fixative, make sure that the fixative employed is compatible with the method of decalcification chosen.
2. Decalcification should be carried out at room temperature and with constant magnetic stirring. While heat accelerates decalcification, it also induces numerous artifacts and thus should be avoided.
3. Do not decalcify longer than necessary, as excessive decalcification will introduce artifacts. To avoid overdecalcification, delicate tissues should be examined every hour and larger tissues examined as established by laboratory protocol.
4. Residual acid will destroy nuclear detail. Therefore, acid decalcification solutions must be removed from bone specimens before they are processed by washing them in water for at least 24 hours.
5. The volume of the decalcification solution should be 10 to 15 times that of the tissue being decalcified. These solutions should also be changed on a regular basis.

Intraoperative Consultation

Important decisions in the operating room are frequently based on intraoperative consultation with a pathologist. These intraoperative consultations often require the rapid microscopic examination of fresh tissue. This examination can be accomplished either by the preparation of cytologic slides or by the preparation of histologic slides using the frozen section technique.

Cytologic slides can be prepared from fresh specimens by one of three methods. Impression smears are produced by touching a microscopic

TABLE 2-2. Acid decalcification methods.

Decalcification method	Tissue	Comment	Advantages (+) and disadvantages (−)
Acid hydrolysis, 3% HCl	Routine decalcification of cortical bone, large thick bone, ossified cancellous bone	Compatible with most routine and special staining methods May interfere with immunoperoxidase and other special studies Method of choice for large specimens	± Immunohistochemistry − Swells cells − Overdecalcification results in extreme eosinophilia
Acid hydrolysis, 5% HNO_3	Routine decalcification of cortical bone, large thick bone, ossified cancellous bone	Compatible with most routine and special staining methods but yields substandard results	− Damages nuclear detail − Immunohistochemistry − Swells cells
Acid hydrolysis, 5% formic acid	Light decalcification of delicate tissue, bone marrow, bones of inner ear	Compatible with most routine and special staining methods Method of choice for small specimens	+ Immunohistochemistry + Good preservation of nuclear detail

slide to the cut face of the tissue. This procedure is also known as a touch preparation. The slide can be air dried or immediately fixed in alcohol for subsequent staining. The crush preparation is performed by taking a small (1-mm cube) piece of tissue and crushing it between two glass slides. The crush preparation can be fixed or air dried, then stained. Extremely hard tissues can be *scraped* with a sharp blade and the scrapings drawn across another slide in much the same way that a blood smear is prepared. Each of these techniques can be used on different tissues with varying degrees of success in terms of preparation artifacts and quantity of cells obtained. For example, crush preparations work best on very soft tissues, while scrapings are needed on very firm tissues.

The frozen section is another procedure frequently used in intraoperative consultations. The details of preparing a frozen section vary greatly from one laboratory to another; however, one should become familiar with a few general concepts.

The first step is to select and prepare a piece of the specimen to freeze. Select a section that demonstrates the interface of the lesion with normal tissue, and try to avoid fatty or calcified tissues. The section should not be greater than 2×2 cm, and wet tissue should be gently blotted dry to avoid the formation of ice crystals. Very small pieces are easily lost in opaque embedding medium, so they should be stained with a drop of eosin or India ink before sectioning.

The second step is to freeze the tissue. Tissues are usually frozen on a tissue chuck in freezing medium by immersion in liquid nitrogen. This technique will lead to the formation of ice crystals, which can distort the histology. Some laboratories therefore prefer to use either refrigerated units with isopentane or cryostats equipped with specialized heat extractors.

Once the specimen is frozen, it is ready for sectioning. A variety of sectioning artifacts can be reduced with a few simple tricks. For example, sections that have been overly frozen will crumble when sectioned. In these cases, simply warm up the block by pressing a gloved thumb firmly onto its surface. (Be careful not to cut your finger on the cryostat blade.) Inadequate freezing, on the other hand, will cause the sections to stick and bind. In these cases, the specimen can be frozen to the appropriate temperature using a cooling spray, such as Histofreeze. Lines and knife marks can be avoided by using a clean and extremely sharp blade. Finally, loosely set screws can contribute to vibration artifacts, so if the sections look like corduroy pants, tighten all of the screws holding the block.

Once the section has been cut, it should be placed on a glass slide, fixed, and stained. A variety of stains are employed in different laboratories (see earlier for the H&E technique); but whichever stains are used, remember to take your time and follow the staining protocol. Too frequently, staining procedures are rushed, and the slides to be stained barely touch the staining

solution. The resultant slides can be impossible to interpret or, ironically, may take longer to interpret than a slide stained correctly. Also, when one rushes, one often transfers solutions from one Coplin jar to the next. As a result, solutions are contaminated, and the quality of subsequent frozen sections is diminished. This problem can be reduced simply by touching the edge of the slide to the edge of the jar before transferring the slide to a new solution. Another problem encountered during staining is that tissue can fall off of the slide. If this happens, try using sialinated or other specially treated slides. The ultimate goal in preparation of a frozen section is to render a timely and accurate intraoperative diagnosis. Remember to save the piece of tissue that was frozen so that it can serve as a frozen section control for diagnostic and quality control purposes.