CHAPTER 1

Tissue Procurement, Processing, and Staining Techniques

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It is an unfortunate reality that many pathologists have only a rudimentary knowledge of the effects of surgical technique and tissue processing on the final results that will be obtained in stained microscopic sections. All too often, one is faced with a sample that has been obtained crudely, fixed badly, or mishandled in the histology laboratory, making morphologic interpretation of it needlessly complex. These faults typically occur not through willful neglect of proper methodology but rather because of ignorance of the sequence of steps that constitute the science of histotechnology. Most trainees in pathology are not well versed in the details of this laboratory discipline, making them totally dependent on the expertise of their technicians, or the lack of it.

Accordingly, this chapter will provide an outline of recommendations for the procurement and subsequent handling of histopathologic specimens. Potential reasons for poor results are also included.

BIOPSY TECHNIQUES

The specific procedures that are used in performing biopsies of clinical lesions are usually left to the discretion of the attending clinician. This provision is not a problem if the operator has been adequately educated on the specific advantages and disadvantages of various techniques, as applied to specific diseases. However, it may prove to be a disaster if the surgeon is inexperienced in such matters. Conventional or enzyme histochemistry, immunohistology, or electron microscopy—all of which are greatly affected by nuances in tissue preservation—may be necessary in some instances to obtain a firm diagnosis. Because the clinician may not be able to anticipate these possibilities before obtaining the tissue sample, a predetermined routine should be followed in doing so (1).

There are basically four generic categories of procedures that may be used in any given case. These include punch biopsies, using circular cutting devices of several sizes; partial or complete excisions done with a scalpel; electrosurgical excisions; and laser-mediated biopsies. In choosing one of these options, the operator should be cognizant of the two opposing "forces" that affect his or her final decision on this matter. On the one hand, the patient is often preoccupied with the cosmetic effects of a biopsy, and this typically induces the surgeon to limit the size of the sample as much as possible. The opposing consideration is represented by the degree of difficulty with which the microscopic diagnosis is made by the pathologist—a factor that is often predictable by the amount of material that will be required to study the disease process adequately.

The cardinal rule to be remembered on this topic is that a properly done biopsy is virtually never cosmetically deforming, if it can be accomplished in an outpatient setting by a competent operator. In contrast, specimen inadequacy and artifactual changes in tissue are problems that relate to faulty procedure, and these account for the great majority of diagnostic obstacles that pathologists encounter. There is nothing quite so aggravating for the clinician (and the patient) as to be informed that a second biopsy will be necessary because of these deficiencies, causing additional expense and anxiety.

As an example of these contentions, it is well known that malignant hematolymphoid proliferations and certain metastatic carcinomas are composed of extremely fragile cells that are exquisitely susceptible to the compressive or shearing effects of some biopsies (Figure 1.1) (2). Moreover, it is probable that several cubic millimeters of tissue will be necessary for the complete pathologic characterization of such lesions. Hence, a small biopsy specimen would be predictably unsuitable in these circumstances. When in doubt, the clinician should contact the pathologist *before* the procedure is done and inquire about recommended handling of the tissue sample and its minimally acceptable size based on the likely diagnostic possibilities.

Other procedures causing reproducibly detrimental physical effects on tissue specimens are represented by

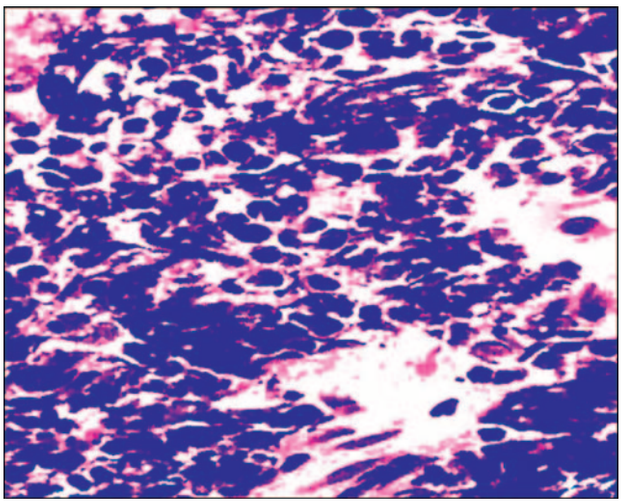


FIGURE 1.1: Smudging or "crush" artifact results from the mishandling of tissue from fragile neoplasms such as lymphoma or small cell carcinoma (shown here).

electrocautery and laser excision. These methods enjoy wide clinical usage at present because of their ease of performance and the limitation of surrounding tissue damage that they afford. Nonetheless, lesional cells in the specimen are often rendered unrecognizable because of widespread thermal coagulation, precluding histologic interpretation altogether. It should therefore be obvious that cauterizing techniques must be avoided for diagnostic purposes. Several adjunctive pathologic studies require the availability of specimens that have been handled in a special manner (Table 1.1). Again, these can be obtained prospectively following preprocedural consultation with the laboratory.

IDENTIFICATION AND ORIENTATION OF THE BIOPSY SPECIMEN

There is nothing quite so exasperating for the pathologist as to receive a specimen that is unoriented and for which no anatomic location is given on the request form for pathologic examination. A lack of meaningful clinical history or a failure to list potential clinical diagnoses often compounds such omissions. These problems usually cannot be solved by the pathologist and typically require a laboratory visit by, or a telephone conversation with, the responsible physician. In many instances, it would be medicolegally dangerous to attempt a morphologic interpretation in the absence of such information. On occasion, a specimen may be received that is so poorly labeled that the identity of the patient is even in question. Such a submission should never be accepted by the laboratory unless the clinician is willing to provide written documentation verifying its origin and accepting exclusive legal responsibility for its interpretation.

If a lesion is a suspected malignancy for which a diagnostic biopsy is also intended to be a complete excision, the clinician should provide some means of identifying the superior, inferior, medial, and lateral borders of the tissue sample. This can be accomplished by attaching sutures of differing lengths or types to the specimen, and sending a corresponding "map" of the tissue to the laboratory along with the pathology request form (3). Alternatively, indelible (e.g., tattoo) ink of various colors can be affixed to the borders of the specimen and identified accordingly (Figure 1.2). As a minimal requirement—for example, in very small excisional biopsies—at least one pole of an elliptical or circular tissue fragment should be labeled by such means.

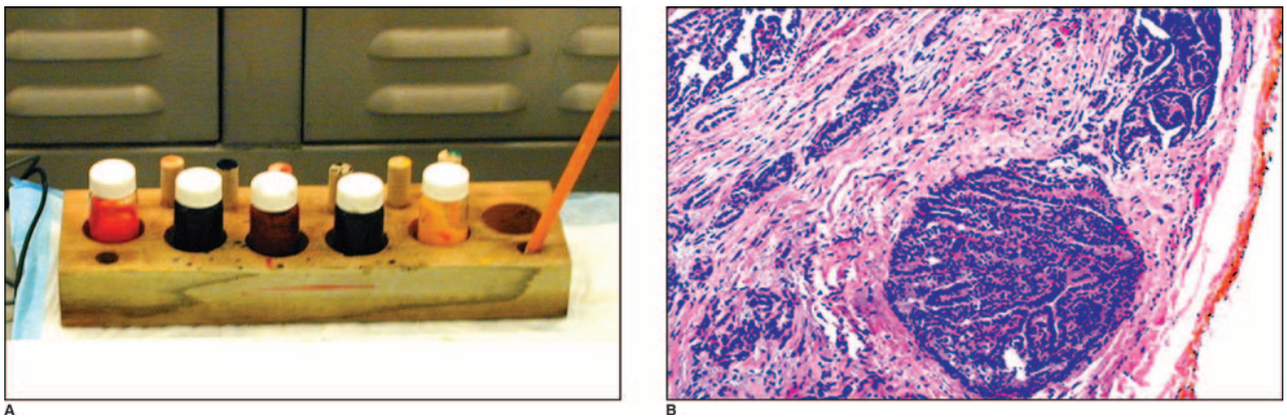
TABLE 1.1: Specimen Processing

Pathologic Technique	Recommended Fixative	Processing Time	Comments
Conventional histology	NBF or FA*	1 day	Tissue should be sectioned at 2–3 mm for good fixation
Immunohistology	NBF or FA**	2–3 days	Technique can be applied to frozen or fixed sections
Electron microscopy	2% phosphate-buffered glutaraldehyde	3–4 days	Tissue must be minced into 1- or 2-mm cubes
Immunofluorescence	None, if tissue is flash frozen; 95% ethanol or acetone for touch preparations; Michel's medium for transportation	1–2 days	Tissue can be held in Michel's medium for up to 48 hours. Frozen tissue must be kept at −70°C until use
In situ hybridization	NBF or FA for DNA studies; frozen tissue preferred for RNA studies	1 week	DNA studies can be done on frozen or fixed tissue

NBF = neutral-buffered 10% formalin, FA = NBF-ethanol (50%:50%).

* Tissue for routine histology can be fixed in B5 or Bouin's solutions to improve nuclear morphology, but these preservatives require special processing and compromise immunohistology.

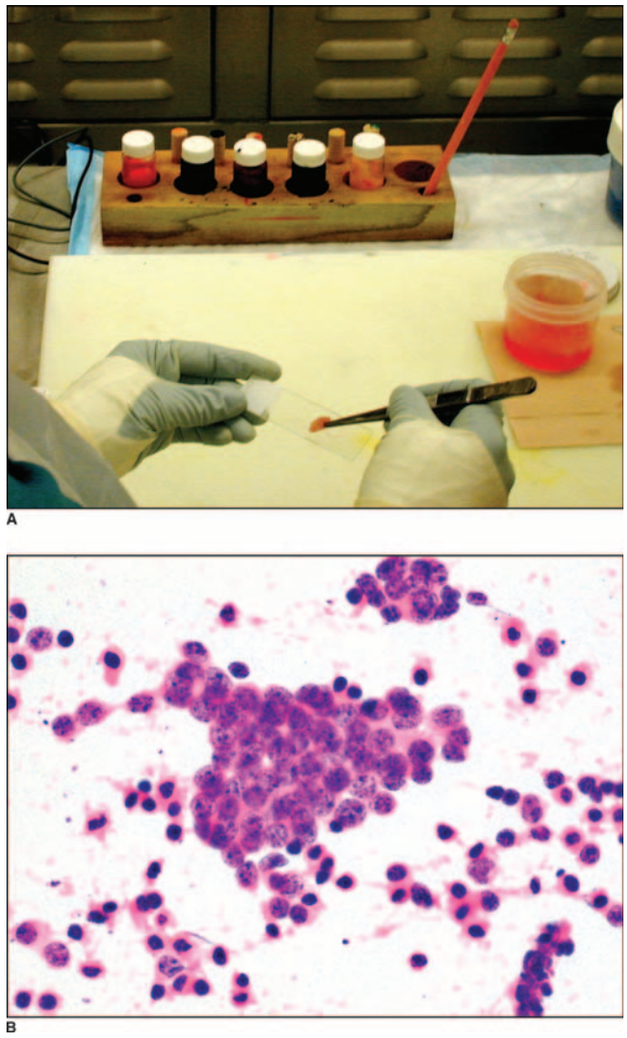
** Certain tissue antigens (e.g., light chain immunoglobulins) are detectable only by frozen section immunohistochemistry.



A B

FIGURE 1.2: *Gross room inking stations (A) contain indelible inks of several colors, which can be applied to specimens with cotton-tipped swabs and fixed in place with Bouin's solution. The ink can then be seen in an FS (B) or permanent section.*

The clinician should be discouraged from attempting to prosect the specimen further before it is examined by the pathologist, except in very well-defined settings. When they are improperly performed, transections of small biopsy samples often confound subsequent orientation steps and may mechanically damage the lesion that is intended for study. The only acceptable reason for undertaking further clinical manipulation of the tissue sample is that of preparing cellular "touch" preparations in examples of suspected hematolymphoid disease. The latter can be obtained if the operator bisects a lesion at its bulkiest point and touches the cut surface of the tissue gently to glass slides in a serial fashion (Figure 1.3) (2). When this is done, special care should be taken subsequently to orient both halves of the resulting two-part specimen for the pathologist. Moreover, all air-dried or fixed touch preparations must be labeled with the patient's name, his or her date of birth or medical record number, and the date on which the procedure was performed.



A B

FIGURE 1.3: *Touch preparations can be made from fresh tissue by serially touching a cut surface from it to adhesive-coated glass slides (A). The touch imprint sections can then be air dried and stained with Romanowsky dyes, or briefly fixed in alcohol and stained with H&E (B).*

PREPARATION OF FROZEN SECTIONS

Intraoperative consultations, generically termed "frozen sections" (FSs) by many surgeons, are often requested in treating presumed or proven malignancies (3,4). Procedural aspects of the FS method are familiar to all anatomic pathologists, but these will be reviewed briefly in this section.

The purposes of obtaining FS examination are twofold; it may be used to secure a rapid diagnosis for a lesion with unknown histologic attributes, or the technique may be employed to confirm that margins of excision are uninvolved by the pathologic process in question. Because of the potential distortion of morphologic detail that this procedure may induce, the first of the cited applications is not one that should be used frequently. With respect to the analysis of excisional margins, the operator must be certain to supervise the orientation and labeling of all specimens,

as outlined above. This makes the availability of indelible ink an absolute requirement.

Following such steps, one must be certain that the tissue sample is small enough to assure rapid and uniform freezing, and ease of sectioning with the cryomicrotome (cryostat). The specimen is usually placed in a small pool of gelatinous, water-soluble mounting medium (e.g., "optimum cutting temperature" medium; Cryogel^{R}) that has been applied to a precooled Teflon or metal "chuck." After making sure that the tissue is properly oriented on the flat surface of this implement, it is then totally covered with additional mounting medium, fashioned into a circular pledget. Immediately thereafter, best results are obtained if the chuck is immersed in a bath of isopentane suspended in an outer container of liquid nitrogen. These devices are available commercially, and they allow for virtually instantaneous freezing of the mounting medium with minimalization of ice crystal formation. The latter eventuality is undesirable because entrapment of ice in the specimen (caused by slowly decreasing temperature) will cause significant distortion (Figure 1.4) and may interfere with microscopic interpretation. For this reason, the utilization of metal cooling "plates," which are incorporated into many cryostats by their manufacturers, is not recommended as a means whereby initial freezing is accomplished. However, these plates are acceptable for maintaining the chucks in a frozen state while sections are being cut.

The microtome in any cryostat must be set in such a manner that uniform sections of reproducible thickness (approximately 5 μm) can be prepared. Regular maintenance regarding the sharpness and integrity of microtomy blades is essential to this process. After "facing" the frozen block with the blade—to obtain a smooth, flat tissue surface—the operator cuts a "ribbon" of several individual sections that can be kept flat by manipulation with a camel-hair brush or with a Teflon-coated panel. These are then apposed to acid-cleaned glass slides that have been kept at ambient temperature, causing the tissue to adhere to them quickly. To eliminate concerns about the subsequent loosening of this bond, slides that have been precoated with albumin, poly-L-lysine, or a chrome-alum gel may be utilized (5).

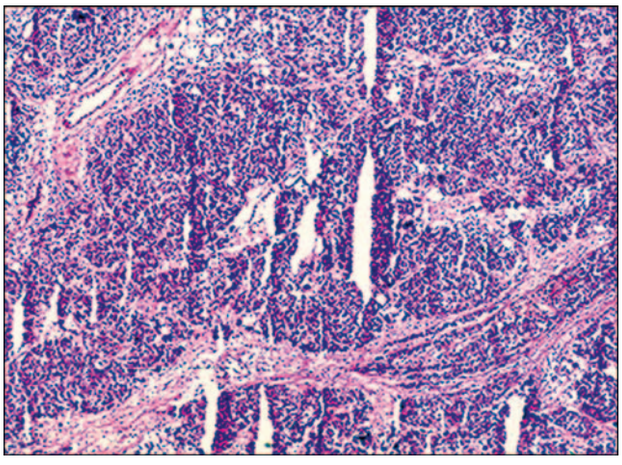


FIGURE 1.4: If ice crystals are allowed to form in tissue during the freezing process in a cryostat, linear defects will appear in the final FS.

Most FS laboratories employ a brief (30–60 second) fixation step immediately after mounted sections are prepared, in Copland jars containing absolute acetone or 95% ethanol. The slides may then be stained with hematoxylin and eosin (H&E), a "polychromatic" or metachromatic reagent such as methylene blue, or other reagents. Following dehydration in graded alcohols and xylene, a synthetic mounting medium is placed over the tissue, and a glass coverslip is applied. Addition of a few drops of xylene to the mounting medium will slightly lessen its viscosity and help to prevent the entrapment of air bubbles under the coverslip.

Alternatively, one may wish to keep some unstained FSs for future studies. This aim is best served by removing slides from the acetone or alcohol fixative and placing them promptly in a freezer at −20 or −70°C. These can be kept in such devices indefinitely for further analysis at a later date.

Specific problems connected with poor microtomy technique will be considered subsequently in this discussion. However, the most common difficulty that is seen in the FS area can be ascribed to improper calibration of the cutting interval between successive sections. Overly thick sections may result in consumption of the tissue before a suitable slide is obtained for microscopic examination; in contrast, it is extremely hard to obtain very thin sections without causing them to fold on themselves or shred. Thus, it is essential for the cryostat to be checked frequently to make certain that it is set up properly from a technical viewpoint. Also, there is no substitute for practice and experience on the part of the operator, in regard to preparation of optimal FS slides. The labeling of specimens used for FS examination should be no different than that used for other samples. The remnant tissue should be placed in a plastic cassette that is suitably inscribed with the accession number of the case (preferably using a CasMark^{R}-type labeler) and kept together with corresponding paperwork for transmittal to the histology laboratory. Under no circumstances should unlabeled frozen tissue be allowed to accrue in the FS laboratory, lest disastrous errors in identification occur.

FIXATION OF SPECIMENS

Questions that are often asked of the pathologist concern the choice of one fixative solution over another for the preservation of various cutaneous specimens. There is no "universal" fixative in pathology because tissue samples may be used for an ever-growing number of investigative analyses, many of which demand that special processing

measures be applied in order to procure optimal results. Selected immunohistologic studies, electron microscopy, and genotypic assessment represent three advanced modalities of pathologic evaluation that are associated with specific fixation requirements. Laboratory specialists are continuing to develop procedural modifications to lessen the need for such provisions, but they still do exist.

In the most optimistic of scenarios, it would be best to submit all biopsies in their fresh state in physiologic saline solution, and for the pathologist to subdivide these specimens into several parts for future diagnostic eventualities. Nevertheless, this is often not practical for two main reasons. First, outpatient specimens are commonly submitted over long distances from the pathology laboratory, increasing the likelihood that unfixed tissue will undergo autolysis before it is received. Second, many biopsies are limited in size, making judiciousness in the selection of special studies an important point. The latter issue again emphasizes the wisdom of preprocedural consultation with the pathologist, if unconventional evaluations are desired.

Fixatives Used for "Routine" Histopathologic Examination

In the great majority of cases, the clinician requesting histologic examination of a biopsy is interested in a "traditional" interpretation based on microscopic findings as seen with the H&E stains. With this stipulation in mind, most laboratories have advocated the use of formalin as the fixative of choice. Nonetheless, the following sections will briefly review the chemical characteristics of preservative solutions in a broader sense, so that exceptions to the above-cited situation may be addressed.

General Considerations

The preservative effects of certain chemicals have been recognized for thousands of years, dating back to the ancient Egyptians. On an empiric basis, therefore, various fixatives have been employed to preclude bacterially mediated putrefaction of human tissues since the inception of pathology as a discipline.

In the past century, detailed studies of these agents have elucidated the probable mechanisms responsible for these beneficial effects (6–10). In addition to antibacterial effects, fixatives also enhance the differences in refractive indices between dissimilar tissue constituents, allowing for greater resolution upon light microscopy. Moreover, they augment the affinity that chemical dyes have for particular cellular elements. It is now known that chemical fixatives may be divided into two broad categories—coagulating and noncoagulating—with respect to their effects on proteins, which form the framework of virtually all cells. Further subdivision into aqueous and nonaqueous agents, as well as additive or nonadditive preservatives, is also possible (11).

Noncoagulative fixatives are the most widely used, and these include formaldehyde (called *formalin* when prepared in aqueous dilution and *paraformaldehyde* when employed in polymeric form), glutaraldehyde, acetic acid, potassium dichromate, and osmium tetroxide. In contrast, acetone, alcohols, chromium trioxide, mercuric chloride, and picric acid exemplify the *coagulative* preservatives. In the process of denaturation and coagulation, a network of altered protein is formed in tissue; in contrast, noncoagulative agents act to produce a stable intracellular "gel." Acetone and alcohol are the major nonaqueous reagents, with most others being soluble in water. *Additive* fixatives react with tissues by combining with them chemically, whereas nonadditive reagents (primarily alcohols and acetone) do not.

Various combinations of these chemicals [e.g., formalin-alcohol or Carnoy's solution (a mixture of ethanol, chloroform, and acetic acid)] are sometimes utilized as fixatives that are intended to augment the stainability of predefined tissue components. Moreover, metal salts—such as those containing zinc and mercury—may be added to aqueous solutions, as in zinc-formalin or "B5" fixative (a mixture of mercuric chloride, sodium acetate, and formalin). The apparent effect of the latter agents is to stabilize complexes that are formed by nucleic acid and protein, yielding improved preservation of nuclear detail. By convention, many fixatives are named for the laboratory investigators who devised them. Thus, one may encounter such designations as Bouin's, Hollande's, Zenker's, Helly's, Zamboni's, Orth's, and Carnoy's solutions. Most of these are mixtures of chemicals in different classes or showing differing effects on proteins, with or without metal salts. Selected reagents in this list will be alluded to later in this discussion.

One important concept to be borne in mind is that *all* fixatives induce chemical artifacts in tissue sections. This effect has two potential ramifications for pathologists. First, we all become inured to the artifacts that we are accustomed to seeing with routine use of a particular preservative solution; indeed, we may even rely on such changes as diagnostic features. Changing the fixative one uses will also alter the tissue artifacts, often leading to interpretative confusion with any given staining method. Second, one artifact produced by a preservative may be desirable, whereas others are detrimental. For example, B5 solution yields excellent nuclear detail on H&E stains, but it virtually destroys the integrity of some cellular proteins that may be the targets of immunohistochemical studies (12). Lastly, the optimal period of fixation varies greatly from one solution to another; tissue placed in formalin may be allowed to remain in it for days with no compromise of morphologic features, whereas specimens in B5, Zenker's, and Bouin's fixatives *must* be transferred to other chemical solutions after predefined periods of time to avoid a serious loss of cellular definition (7,8). Thus, the ultimate choice of a preservative solution is not one to be made indiscriminately.

Specific Fixatives

Formalin: Formalin represents a 37–40% aqueous solution of formaldehyde, the latter of which is marketed commercially in the United States. Because the former reagent is characteristically used at a 10% dilution, the final formaldehyde concentration is 3.7–4%. Various other chemicals have been added to formalin to alter its stability and preservative capabilities, including calcium chloride, calcium carbonate, ammonium bromide, sodium chloride, sodium phosphate, sodium hydroxide, and absolute ethyl alcohol. Among these mixtures, that consisting of formalin, distilled water, and monobasic/dibasic sodium phosphate is the most widely employed and is known as "10% neutral-buffered formalin" (NBF). *Paraformaldehyde* is a polymerized form of formaldehyde admixed with methanol; it is generally employed as a fixative for specialized immunohistologic procedures, particularly when combined with periodate and lysine ("PLP" solution) (12,13).

Although it is a general-purpose fixative and yields good morphologic detail when prepared properly, NBF does have some disadvantages in tissue pathology. First, any solution containing formaldehyde is potentially carcinogenic, and levels of formalin vapor in the ambient air of the laboratory must be measured regularly by governmental mandate. The maximum permissible exposure limit for any individual employee is 1 part per million over an 8-hour period, as established by the Occupational Safety and Health Administration (14). Second, poorly prepared NBF, which has been buffered erroneously and has a pH outside of the physiologic range, may cause unwanted precipitates of "black acid hematin pigment" in tissue sections. The latter has a dark particulate appearance, and may simulate microorganisms on a histologic slide. These two possibilities can be distinguished through the use of polarization microscopy because hematin pigment is birefringent, whereas microbes are not (11). Third, NBF that is allowed exposure to ambient air for prolonged periods of time (as with large "batches" that are diluted for use in the gross laboratory) will develop high levels of formic acid. The latter is detrimental to protein substructure and may accentuate the formation of methylol bonds between polypeptides. This effect can "mask" proteinaceous epitopes that correspond to the targets of immunohistologic antibody reagents (15). Lastly, formalin has a limited capacity for penetration of bulky pieces of tissue, and specimens fixed in it must be no thicker than 4–5 mm.

Despite these drawbacks, formalin is inexpensive and widely available, and is therefore ubiquitously employed as the fixative of choice for clinical specimens. The above-cited failings of this preservative can be prevented by careful technique in its preparation, adherence to environmental monitoring requirements, and application of proper prosection and fixation techniques for the submission of tissue sections. Some laboratories prefer to use NBF ethanol (mixed in equal volumes) because it affords a greater degree of tissue penetration than formalin alone.

B5/Zenker's/Helly's Solutions: B5, Zenker's, and Helly's solutions were introduced because of their superiority over NBF in the preservation of nuclear detail (7,8). They are fixatives based on the inclusion of mercuric chloride, with or without sodium acetate, potassium dichromate, sodium sulfate, acetic acid, and formaldehyde as additional constituents. Because of the excellent morphologic detail that is achievable with these solutions, many laboratories prefer them for the routine preparation of H&E-stained sections. Nevertheless, there are three distinct disadvantages of B5, Zenker's, or Helly's reagent, as compared with NBF. Tissue sections must be removed from the former three fixatives after no more than 8 hours and placed into 70% ethanol; if this is not done, specimens will become extremely brittle and virtually impossible to section (11). Also, the presence of mercuric chloride will cause deposition of pigment in microscopic preparations, which must be removed with iodine before final staining procedures are done. Lastly, mercury-based solutions are powerful coagulating agents and therefore damage many cytoplasmic proteins. This effect commonly renders tissue sections unsuitable for a variety of immunohistochemical studies (12).

Bouin's Solution: Bouin's fixative is again based on formaldehyde as a major component, together with picric and acetic acids in aqueous solution. Like B5, this reagent affords excellent preservation of nuclear morphology but suffers from failings pertaining to brittleness of tissue, pigment deposition, and adverse effects on cytoplasmic polypeptides. In addition, Bouin's-fixed specimens acquire a yellow color (because of the effects of picric acid) that must be removed by postfixation washing in alcohol and lithium carbonate. Bouin's fixative is preferred for visualization of delicate mesenchymal tissues because of its superior differentiating abilities in regard to these elements (11). Accordingly, some "stromal" special stains (such as the Masson trichrome method) are best performed on specimens preserved in this solution.

Acetone and Alcohols: Acetone and alcohols are rapidly acting fixatives with good penetration of tissue. They also afford better preservation of some cytoplasmic enzymes than formaldehyde-based solutions do, in paraffin sections. However, two major disadvantages attend the use of these organic reagents. They cause striking shrinkage of tissue because of their dehydrating effects, thereby altering morphologic details appreciably. Also, acetone and methyl or ethyl alcohol are relatively expensive, and they require special storage and inventory procedures because of possible use by laboratory workers as inebriants. In current practice, these agents are usually applied only in the fixation of

touch preparations and are not commonly utilized in the processing of biopsy specimens. Similar comments apply to Carnoy's solution, which is constituted by ethyl alcohol, chloroform, and acetic acid.

Decalcifying Solutions: Some biopsy samples may contain obvious foci of calcium salts, as suggested by anatomic location, clinical findings, or difficulty in performing the biopsy procedure. In these circumstances, two main methods exist for the removal of such minerals from the specimen. One employs simple acids (hydrochloric or nitric), which rapidly solubilize calcium deposits. The other technique is based on the ability of certain chelating agents—such as ethylenediaminetetraacetic acid (EDTA)—to accomplish this task. The second of these methods is much gentler and does not cause the loss of microscopic detail that acid decalcification may incur. Fixation is allowed to progress in concert with decalcification with both acidic and EDTA reagents because they are commercially marketed as mixed solutions containing formaldehyde.

Glutaraldehyde: Glutaraldehyde is similar in chemical activity to formaldehyde; both cause cross-linkage of proteins in tissue (7). However, glutaraldehyde penetrates specimens very slowly, making the size of the tissue sample a critical determinant of fixation with this reagent. Moreover, 2–4% glutaraldehyde (representing the usual working concentration) has a propensity to cause brittleness of specimens that are immersed in it for more than 2–3 hours; transfer to a buffer solution is absolutely necessary after this point. For these reasons, among others, glutaraldehyde is not used often for the preservation of biopsy samples that are intended for light microscopy. However, it is the preferred fixative for electron microscopy, wherein specimens are very small and limited "hardening" of tissue may actually be morphologically advantageous.

Other Factors Influencing Fixation

As outlined by Carson (11), there are several other considerations in the fixation of tissue besides one's choice of preservative solution. These include temperature, size of the sample, the volume ratio of tissue to fixative solution, the duration of fixation, and the pH of the solution.

Recently, the rapid but controlled elevation of temperature with microwave ovens has been utilized as an independent means of fixation, by coagulation of tissue proteins (16). Surprisingly, this process appears to have little if any adverse effect on staining characteristics, even with immunohistologic methods. However, it must be emphasized that careful control is the key to thermal fixation; overheating may completely destroy the specimen if it is allowed to reach an extreme level (e.g., over 65°C). In a more conventional context, there are really no compelling reasons to employ fixative solutions at one temperature versus another.

Specimen size is, in contrast, a potentially crucial factor affecting quality of fixation, and this determinant goes hand in hand with the volumetric relationship between a tissue sample and the solution in which it is immersed. Large, extremely thick specimens will be inadequately penetrated by most fixatives, allowing autolysis to proceed unchecked in their central areas. This problem results in eventual loss of the unfixed foci during microtomy, yielding microscopic sections that resemble doughnuts (Figure 1.5). Because penetration is facilitated by minor thermal or mechanical currents in the fixative solution, large specimens that are covered with an inadequate volume of preservative will predictably be underfixed. An experienced histotechnologist typically detects this problem upon attempting microtomy of the tissue and will "run the specimen back" for more prolonged fixation and reprocessing. However, this consumes additional time and should be unnecessary.

As noted at several points in the foregoing discussion, there is a maximum recommended period of fixation with several preservatives, over which unwanted changes reproducibly occur in tissue biochemistry. Overfixed specimens are difficult to cut and often demonstrate alterations in morphologic definition or antigenic integrity. In contrast, underfixation allows bacterial putrefaction to proceed, similarly damaging the tissue sample. Specimens that are immersed in the most commonly used preservative—NBF—should ideally be processed further within 8–12 hours.

The pH of fixatives is not critical for light microscopy, except that certain unwanted pigmentary deposits may be seen with unduly acidic preservatives. Nonetheless, hyperacidity is extremely detrimental to cellular ultrastructure and also to the maintenance of tissue antigenicity

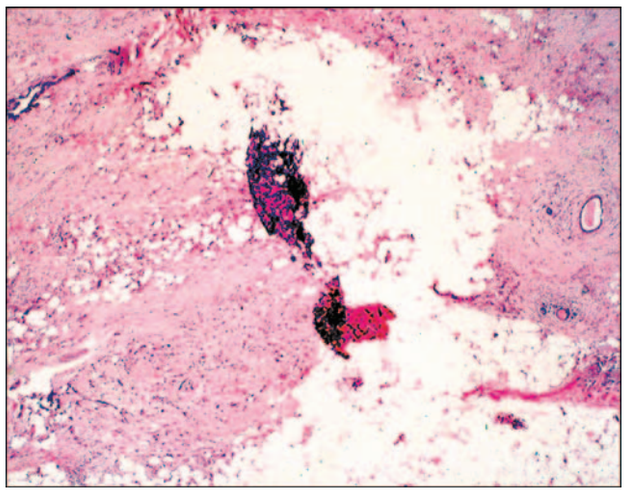


FIGURE 1.5: Inadequate fixation of tissues—especially fatty ones—will often result in loss of the central areas of tissue blocks in final microscopic sections because they "fall out" during processing.

(11,12). For these reasons, it would be wise to control pH within the physiologic range during fixation, in the event that electron microscopy or immunohistology is necessary diagnostically.

Tissue Processing and Preparation of Microscopic Sections

Because most commonly employed fixatives are aqueous in nature, the next step in tissue processing is usually that of dehydration and "clearing" (removal of all water from the specimen). Graded solutions of ethanol are used for this purpose, and these must be changed frequently to maintain their desiccating properties. A variety of clearing agents are available, but the most common are xylene and limonene derivatives. In likeness to the alcohols, such reagents may be contaminated by water with repeated use and should be monitored closely for this problem.

Xylene is inexpensive and does not leave a residue on glassware or other instrument parts in the histology laboratory. In light of these virtues, it is the most popular clearing agent. However, xylene fumes are potentially toxic to technologists, making careful storage, controlled disposal, and environmental monitoring mandatory. In addition, we have found that xylene may damage the protein substructure of certain fragile tissue antigens (12). Limonene-type clearing agents are derived from plants and are therefore biodegradable. They have a strong odor—like that of lemons or oranges—which is alternatively perceived as pleasant or noxious by various people. Other disadvantages of limonenes are that they leave a residue on mechanical tissue processors and may sometimes interfere with the adherence of tissue sections to glass slides. The microtomy of specimens cleared in limonenes has been said to be easier than that encountered with xylene (11).

In the relatively early days of histotechnology, all dehydration and clearing steps were done by hand. Over the past 45 years, however, a variety of automatic tissue processors have been engineered and marketed. These are used widely at present and may be divided into two main groups—"open" and "closed." Open processors mechanically transfer baskets containing tissue cassettes from one "station" (chemical bath) to another, on a computer-driven schedule. The latter may be altered by the operator to change the time of dehydration, clearing, or other steps. Closed instruments vary the solutions to which each specimen basket is exposed by pumping chemicals in and out of fixed chambers, again according to a programmed schedule. In other words, open processors move specimens, whereas closed processors move chemical solutions.

Each of these two types of instruments has advantages and disadvantages. Open processors show a low incidence of reagent contamination from one station to another, but they are subject to the mechanical "hang-up" of specimen baskets in transit. Closed processors do not suffer from the latter drawback, but they are subject to chemical carryover from one reagent pumping step to another. This potentially compromises the dehydration-clearing sequence. On balance, individual experience on the part of technologists and pathologists ultimately determines which type of processor will be chosen.

EMBEDDING AND SECTIONING OF BIOPSY SPECIMENS

The final stations in any tissue processor infiltrate all specimens with paraffin or another wax-based embedding medium. Thereafter, the technologist removes each biopsy (one at a time) from its metal or plastic cassette and proceeds to embed it in a rectangle of additional liquid wax, with attention to the proper orientation of the tissue sample. The pathologist may direct this process by notching or inking one or several surfaces of the specimen (Figure 1.6) and providing a "map" in accompanying paperwork that indicates whether these should be placed facedown, faceup, or in parallel with the lateral aspects of the cassette. Such provisions are usually necessary only with large pieces of tissue. For example, technologists accustomed to handling skin biopsies will, as a matter of routine, orient the epidermis perpendicularly to the bottom of the cassette mold and facing one of its long sides. If several pieces of tissue are included in the same block, these are best arranged diagonally.

The embedding step is a potential source of great irritation (and medicolegal liability) for the pathologist if it is done by an inexperienced or careless laboratory worker. With few exceptions, small biopsy specimens that are oriented improperly cannot be interpreted microscopically (Figure 1.7), necessitating that the block be remelted and

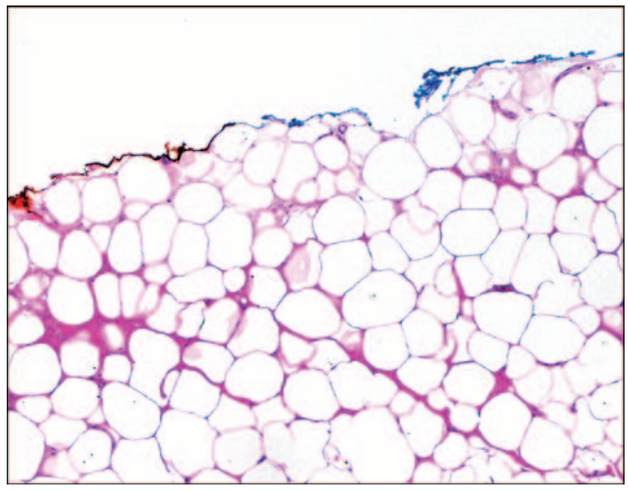


FIGURE 1.6: This microscopic section of a breast biopsy shows the intersection of two anatomic planes that were labeled by the submitting surgeon, and inked with two different colors by the prosecting pathologist.

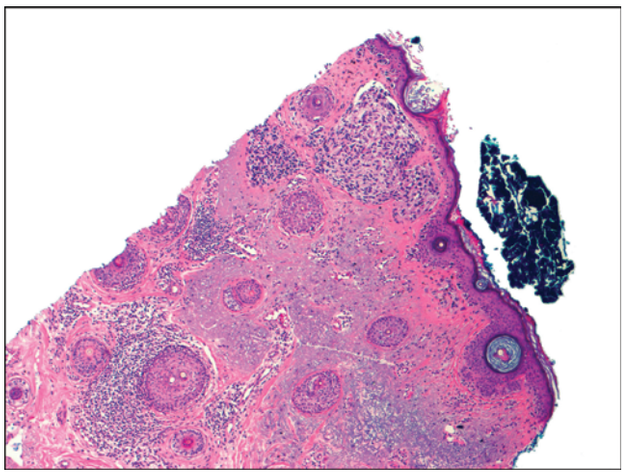


FIGURE 1.7: ***Malorientation of biopsy "tips" or bisected biopsy specimens at the embedding station, as shown here, will compromise the pathologists' ability to evaluate the true specimen margins for tumor involvement. Reprocessing may or may not solve this problem, and every effort should be made to avoid it in the first place.***

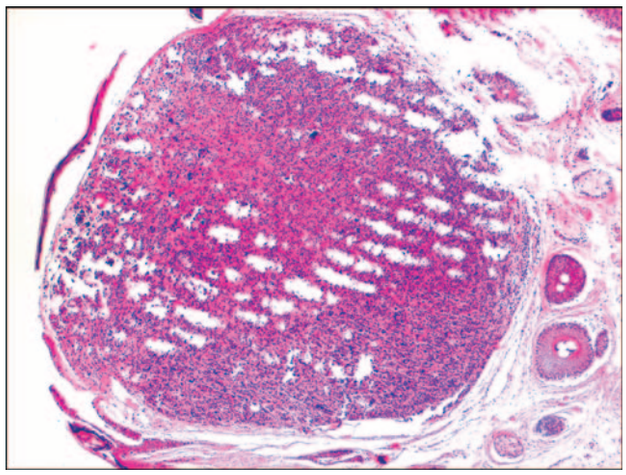


FIGURE 1.8: ***"Chatter" artifact in tissue sections is the result of loose microtome blades or poor microtomy technique.***

re-embedded. This takes time, and in the process of facing the poorly oriented specimen for preparation of initial sections, valuable tissue may be lost (17).

In order to circumvent embedding difficulties, some pathologists have taken to *pre*-embedding small biopsies in agar before they are put in cassettes for fixation. This does assure proper orientation, but agar will not "fix" in the same manner that tissue does, nor will it respond similarly to dehydration, clearing, and infiltration by wax. All these factors may cause the tissue to "pop" free of the surrounding agar after embedding and during tissue sectioning, defeating the purpose of the agar impregnation step altogether. Therefore, we do not advocate this procedure, rather preferring to educate technologists on the details of orientation during wax embedding. Even a very small biopsy can be appropriately configured in the wax block, with the use of a magnifying lens or dissecting microscope.

Paraffin is still the most widely utilized embedding medium, but some laboratories have opted to employ "Carbowax" as a substitute. The latter compound is a water-soluble wax, making dehydration and clearing of the tissue unnecessary and allowing for direct infiltration of formalin-fixed tissue with embedding medium in the tissue processor (11). This element of simplicity is attractive, but Carbowax has its drawbacks. One concerns the dissolution of the embedding medium when microtomized tissue ribbons are placed in a water bath prior to mounting them on glass slides. This unwanted eventuality makes it difficult for the technologist to keep the tissue section flat, resulting in undesirable folds in the final stained slide. Second, we have noted irregularities in antigen preservation when Carbowax-embedded tissues are studied immunohistologically. The temperature of paraffin or Carbowax stations in the tissue processor, and at the embedding center, must be monitored closely. Overheating the wax will cause unwanted thermal artifacts in the tissue and compromise its cellular detail. Excessively cool wax fails to infiltrate the specimens adequately.

Another class of embedding compounds that is presently in vogue in some centers is represented by polymeric plastic resins such as glycol methacrylate or epoxy. Disadvantages of these compounds include the necessity of cutting corresponding tissue sections with a glass or diamond knife microtome, and the requirement for a transitional fluid, such as propylene oxide, to embed the tissue after dehydration and clearing (11). Moreover, plastic sections are difficult to stain with the same intensity as that seen in paraffin-embedded preparations. The main advantage of plastic media is that extremely thin, flat sections may be prepared by experienced microtomists, providing exquisite cellular detail. In addition, some enzyme-histochemical staining methods that otherwise require the use of FSs are possible with specimens embedded in epoxy or glycol methacrylate.

Histomicrotomy is a seemingly straightforward process, representing the cutting of serial paraffin-embedded sections with a tissue microtome. Nevertheless, this technique has many hidden traps that relate to the proper maintenance, calibration, and orientation of cutting blades; preparation of paraffin blocks; and dexterity of the technologist. Microtome blades that are dull loose or nicked will produce "chatter" or "venetian blind" artifacts in tissue sections (Figure 1.8). In addition, the "clearance angle" (between the tissue block and the microtome knife) is crucial to good technique. It should be approximately 3–8°. If the angle is too narrow, alternately thick and thin sections are cut, or they are folded on themselves (18–20). An excessive clearance angle causes chattered or otherwise

hideous sections and may preclude the ability of the technologist to obtain a tissue ribbon. Even worse are the effects of loose microtome blades or tissue blocks in the microtome chuck. These deficiencies may shatter the paraffin block entirely or deeply groove the tissue specimen. A block that is mounted crookedly in the microtome chuck will produce irregular ribbons or cause individual sections in the ribbon to break free from one another.

Regardless of whether one uses paraffin or Carbowax as an embedding medium, there is still a need to refrigerate tissue blocks before microtomy is attempted. This step hardens the wax slightly and allows for crisp sections to be cut. Warm blocks will yield wrinkled ribbons or cause successive sections to anneal to one another. In addition, failure to moisten the surface of blocked tissue suitably before cutting it yields an excessive number of knife marks or fragmented sections. The technologist can simply rub a wet finger over the block several times prior to microtomy, if the specimen is small. If it is large, and particularly if the tissue is heavily cornified, a wet piece of cloth or cotton soaked in 5% ammonium hydroxide may be applied for 2 or 3 minutes to rehydrate the tissue face (18).

Another problem that is sometime seen at this step is the tendency for ribbons to "fly" onto the knife blade. This is the result of static electricity between the wax or tissue and the metal blade, and also may be avoided by slightly moistening the knife and the block surface before each ribbon is prepared.

MOUNTING OF TISSUE SECTIONS

The wax ribbon of serial tissue sections can be removed from the microtome knife as it is cut, by using a wooden tongue depressor blade. In this process, the operator exerts slight traction on the end of the ribbon, stretching it gradually over the wooden blade, and subsequently depositing it on the surface of a warm water bath at the cutting station. The temperature of such flotation devices should be kept at 5–10°C below the melting point of the embedding wax. If it is too hot, desiccated-looking sections will result; in contrast, cool flotation baths produce excessive wrinkling of the tissue.

To facilitate the process of obtaining a smooth, unwrinkled, paraffinized ribbon of tissue, it can be stretched by slight traction on its ends while floating in the warm water bath. Also, we have found that adding a few milliliters of ethyl alcohol to the water is beneficial in this regard. The ribbon must not be left in the bath for more than 1 or 2 minutes, or spurious overhydration of the tissue will be produced. This effect simulates the appearance of edema fluid microscopically (17). Because tissue sections do not adhere well to untreated glass slides, a bonding agent also must be a component of the water bath. Elmer's-Glue^{R}, albumin, and poly-L-lysine are all suitable additives of this type.

One of the most dangerous of all mistakes in the histology laboratory can take place when mounting sections from flotation baths. Friable tissue may "shed" small fragments that float free on the surface of the water, and these may be inadvertently picked up when mounting slides from subsequently processed unrelated cases. Derisively known as "floaters," these rogue pieces of tissue commonly cause agonizing interpretative problems for the pathologist (Figure 1.9). For example, it is not difficult to envision a small piece of a prostatic carcinoma that may find its way onto slides of another prostate biopsy, a ribbon of which is mounted subsequently in the same water bath. Technologists must be impressed with the tremendous medicolegal liabilities that such a mistake incurs, and they must routinely skim, or otherwise clear, the surface of the water bath between cases. An alternative source of floater-type artifacts is the "tongue blade metastasis," wherein tissue adheres to a wooden applicator stick that is used to float successively prepared ribbons from two different cases (11). Needless to say, this practice is highly inadvisable.

With respect to optimizing the cost of slide preparation, we recommend that as many individual sections as possible should be mounted on one slide, from the same ribbon. It is not difficult for adept technologists to include three to five cuts of a specimen on each slide, arranged in a serial fashion. Also, in light of the limited size of many skin, bronchoscopic, and gut biopsies, it is advisable to save any unmounted paraffin ribbons (with appropriate identification) from such cases for 1 week after they are accessioned. Remounts can be prepared from these directly, without the need for further microtomy of the tissue block.

Finally, the identification of tissue sections must be scrupulously maintained throughout the remainder of their sojourn in the histology laboratory. Such a necessity is assured by having the technologist scratch the case and

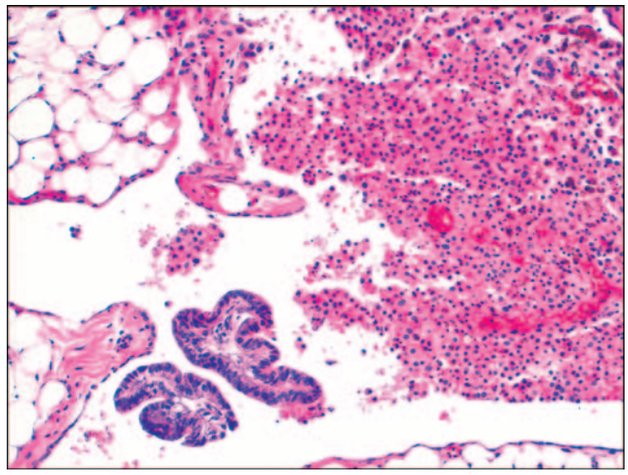


FIGURE 1.9: "Floaters" (lower left) in microscopic sections are unwanted pieces of tissue from other cases. They may cause serious diagnostic mistakes and again should be avoided at all costs.