Chapter 2

The Pathologist's Guide to Fixatives

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Abstract

Proper tissue fixation is essential to ensure the highest level of specimen evaluation. Pathologists and laboratory staff are frequently consulted by clinical counterparts regarding what fixative should be used for different tissues or to enable a diagnosis of a specific condition. It is vital for the patient that the pathologist provides accurate information to ensure proper fixation. Frequently, once a tissue has been fixed inadequately or inappropriately, remedial changes may no longer be possible. Most often formalin is an adequate choice, if not the optimal one; however, there are certain situations when placing the tissue in formalin may limit the ability to reach a definitive diagnosis. It is imperative for pathologists to have the knowledge to communicate which fixative is optimal. Furthermore, as we move into a world of personalized medicine, where ancillary testing has both diagnostic and specific therapeutic implications, knowledge about how different fixatives affect immunohistochemistry, cytogenetics, and molecular studies becomes even more significant. This chapter provides practical information regarding common fixatives, their mechanism of action and optimal uses.

Key words Fixatives, Tissue processing, Anatomic pathology

1 Introduction

Fixation is the critical step in processing tissues. Following tissue removal from the body, autolysis begins and proceeds more or less quickly depending on many factors, including the level of enzymes or microorganism present in the tissue. The purpose of the various fixatives is stabilization of those enzymes and other tissue proteins and disabling microorganisms, thereby arresting autolysis, with the goal to preserve the tissue as close to the in vivo state as possible. Using the appropriate fixative is necessary to ensure the most significant histologic features are highlighted while not interfering with or precluding ancillary testing that may be required.

Fixatives can be classified in different ways depending on their mechanism of action. In the most general terms, there are physical and chemical methods of fixation. Physical fixation processes includes augmenting a fixative with heat or microwaving.

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Cryopreservation is another physical method. Heat preservation and cryopreservation are rarely used on diagnostic tissue specimens [1]. While microwaving has become common practice in the histology laboratory, it is used primarily in tissue processing and will not be discussed here. The chemical fixatives can be classified a few ways, the simplest being those that are additive and form cross-links and those that denature, most commonly accomplished by dehydration [1]. The additive fixatives literally add themselves onto the tissue via chemical bonds, with the extent of bond formation varying considerably. Many of the fixatives routinely used in pathology are additive solutions, including formaldehyde, zinc sulfate, glutaraldehyde, picric acid and mercuric chloride [2]. The non-additive or denaturing fixatives act primarily by dissociating water molecules from the tissue proteins resulting in a change in the tertiary structure. For example, methyl and ethyl alcohol coagulate protein but are not added to the tissue. This causes water-soluble proteins to become insoluble, a process that is largely irreversible [3].

Numerous factors affect tissue fixation. The most significant of these include: time of fixation, temperature, permeability, volume ratio, specimen dimension, pH and osmolality [2, 4, 5]. Recommendations for use and possible negative effects of each are summarized in Table 1. These factors, along with the appropriate choice of fixative, can significantly impact the ability to make an accurate diagnosis. The remainder of this chapter highlights the common fixatives, optimal use of each, and provides a resource for both pathologists and clinicians to ensure the appropriate fixative is selected for individual specimens. The chapter ends with a table dedicated to tissue requirements in molecular and cytogenetic testing. One of the most frequently asked questions for pathologists today is a "can you do the recommended molecular test on the specimen we gave you?" With the vast array of testing being performed, and the limited training today's practicing pathologists have received in this area, it is often a daunting task to answer this question correctly. Finally, it must be emphasized, as Table 1 indicates, appropriate tissue preparation is critical.

2 Materials

While many of the fixatives used in pathology laboratories are now commercially available, the common formulations are listed for completeness and reference. Unless otherwise stated, the fixative recipes listed are procured from Carson and Hladik's Histotechnology: A Self-Instructional Text [2]. Prepare all solutions at room temperature unless otherwise specified and work in a well-ventilated area wearing goggles, gloves, and lab coat.

Table 1
Factors influencing fixation [2, 4, 5]

Factors influencing fixation	Recommendations	Possible negative effects
Specimen dimensions	3 mm tissue sections are ideal 4 mm maximal thickness	Thicker sections result in incomplete fixation (*see* **Note 1**)
Time of fixation	Varies by fixative: Formalin—requires 6–8 h Bouin's—not more than 18 h B-5—2–4 h then transfer to formalin	Under-fixation can result in tissue distortion and poor staining; over-fixation with some fixatives (alcohols, B-5) can make tissue brittle or result in loss of antigenicity (*see* **Note 2**)
Penetration rate	Depends on the diffusion characteristics of each fixative: Formalin penetrates at about 1 mm/h	Fascia and capsules are naturally occurring physical barriers to fixatives and can dramatically decrease penetration. They must be incised prior to fixation
Temperature	Room temperature is ideal for the majority of tissue fixation and up to 45 °C during processing	Heat increases the rate of fixation but also speeds up autolysis, which can result in poor morphology and staining (*see* **Note 3**)
Volume ratio	Generally accepted as 15–20:1 fixative to tissue ratio	The concentration of active reagent in fixative diminishes as the chemical reaction of fixation occurs. If depleted, fixation will cease no matter how long tissue remains in the fixative (*see* **Note 4**)
pH and buffers	Breakdown of formaldehyde results in formic acid which decreases pH. Buffers help avoid this by maintaining pH between 6.8 and 7.2	Formic acid reacts with hemoglobin to produce formalin pigment that deposits in tissue and can be misinterpreted as microorganisms or other pigments (melanin, iron); this can be removed from tissues in the staining process by short immersion of slides in Lugol iodine
Osmolality	Do not place tissue in water or leave in saline for excessive periods of time If immediate fixation is not possible, refrigerate, place on saline soaked gauze, or immerse in isotonic saline for a short period	Cell lysis can occur if placed in hypotonic solution

1. 10 % Neutral Buffered Formalin (NBF): Tap water (900 mL); formalin (100 mL 37 % formaldehyde solution); sodium phosphate, monobasic, monohydrate (4 g); sodium phosphate, dibasic, anhydrous (6.5 g); mix sodium phosphate (monobasic and dibasic), formaldehyde, and distilled water. Check pH (should be 6.8–7.2). Label with formaldehyde warning and date (*see* **Note 5**).
2. Alcoholic Formalin [6]: 40 % formaldehyde (100 mL); 95 % ethanol (900 mL); 0.5 g calcium acetate (to ensure neutrality). Mix well and label with formaldehyde warning and date.
3. Phosphate-Buffered Glutaraldehyde: $NaH_2PO_4 \cdot H_2O$ (1.9 g); sodium hydroxide (0.43 g); distilled water (100 mL); mix well and add glutaraldehyde, 25 % solution (18 mL). Check pH range (should be 7.2–7.3). Store refrigerated or frozen (0–5 °C), thawing just prior to use (*see* **Note 6**).
4. B-5 stock solution: mercuric chloride (12 g); sodium acetate (2.5 g); distilled water (200 mL). Mix components well. Label and date the container. Immediately before use, make B-5 working solution by adding 20 mL of B-5 stock solution to 2 mL formaldehyde and mixing well. Do not use metal utensils or foil lined lids (*see* **Note 7**).
5. Zenker's stock solution: mercuric chloride (50 g); potassium dichromate (25 g); sodium sulfate (10 g); distilled water (1,000 mL). Immediately before use make Zenker's working solution by adding 95 mL of stock solution with 5 mL of glacial acetic acid and mixing well. Label and date the container (*see* **Note 8**).
6. Alcohol, 95 %: absolute alcohol (950 mL) and distilled water (50 mL). Mix well.
7. Hollande's fixative: copper acetate (75 g); picric acid (40 g); formaldehyde 37 % (100 mL); acetic acid (15 mL); distilled water (1,000 mL). Dissolve chemical successively in the distilled water without heat. Label and date the container (*see* **Note 9**).
8. Bouin's fixative: picric acid, saturated aqueous solution (750 mL); formaldehyde, 37 % (1,000 mL); glacial acetic acid (200 mL). Mix well. Label and date the container.

3 Methods

3.1 Optimal Use of Specific Fixatives

For most tissues, more than one fixative can be used depending on the goals of the study. In fact, the preferred fixative for specific tissues varies from pathologist to pathologist, institution to institution. For example, some laboratories prefer Bouin's solution to

formalin for urothelium biopsies [7] because they believe it offers superior morphologic detail. Similarly, some labs are willing to endure the difficulties of working with B-5 for possible lymphoma cases for the same reason (see Lymph Node chapter). Table 2 highlights the most commonly recommended fixative for different tissue types and acceptable alternatives. The main point is that there are options and the best fixative should be determined by each laboratory. It is only through trial and error that the optimal set of fixatives can be determined for each individual group preference.

3.2 Fixatives for Specific Tissue Types

It is not uncommon to have a clinician call the pathologist to ask, "what should I put this specimen in to make sure I can make a diagnosis of [X]?" The pathologist must be able to appropriately counsel the clinicians as to the cases where formalin is not the correct choice and when it is acceptable. Table 3 summarizes fixative useful in diagnosing both common and uncommon diseases and tissues requiring special handling.

3.3 Molecular Testing and Fixation

As we enter the age of personalized medicine, it becomes increasingly important to optimize tissue collection, fixation, processing and storage of tissues to ensure adequate quality for diagnosis and therapeutic decision making. Maintaining high quality morphology, without compromising the ability to perform immunohistochemistry, in situ hybridization and nucleic acid extraction, is the goal. Neutral buffered formalin remains the primary fixative used in clinical medicine for many reasons (e.g., relatively inexpensive, widely available commercially as well as easily prepared, adequate for most histologic purposes and highly stable) in spite of the fact that its functions as a fixative are marginal for most of these modern uses [11]. Some of these limitations have been overcome with the development of techniques such as antigen retrieval, enabling more successful IHC testing in formalin-fixed tissue. And while fresh, or fresh frozen tissue is still considered the gold standard for providing high quality RNA and DNA [12, 13], the majority of molecular tests performed for diagnostic and therapeutic purposes can now be applied to formalin-fixed paraffin-embedded tissues (FFPE) (*see* **Note 10**). Several factors determine successful DNA extraction for molecular analysis, including: type of fixative, duration of fixation, and the age of the paraffin block [14–16]. It is important to remember that not all tissues are fixed in NBF. Knowing which of the lesser used fixatives provides adequate material for diagnostic and research based IHC and molecular testing is also important. Fixatives resulting in adequate samples and those resulting in unacceptable samples are listed in Table 4.

Table 2
Common fixatives: uses and limitations

Fixative	Primary/recommended use	Limitations
Alcohol	Routine cytology specimens Cases where gout is suspected Fixation for frozen sections, smears, and touch preps	Methanol and ethanol cause cell shrinkage and will make tissue brittle if over fixed
Alcoholic formalin	Completion fixation with incompletely fixed tissue; primary fixative for fatty specimens (allows for easier detection of lymph)	Acidic pH can allow for formation of formalin pigment precipitates
B-5	Hematopoietic and lymphoid tissue	Sections require removal of mercury pigment prior to staining; tissue cannot be stored in this; low molecular weight or no extractable nucleic acid
Bouin's	Gastrointestinal and genitourinary tissue	Slowly removes small calcium and iron deposits; lysis of erythrocytes; low molecular weight or no extractable nucleic acid
Decalcifying solution (acid based)	Large bone sections where future molecular testing is not required	Poor staining with low molecular weight or no extractable nucleic acid Prolonged immersion can completely dissolve specimen
Decalcifying solution (EDTA based)	Bone biopsies for primary diagnosis or secondary diagnosis (metastasis), bone aspirates	
Formalin	Routine processing	Dissolves uric acid crystals; can dissolve breast microcalcifications if fixed >24 h prior to processing; reduced high molecular weight nucleic acids with time Unbuffered formalin can allow for formation of formalin pigment precipitates
Glutaraldehyde	Electron microscopy	Can cause false positive PAS staining [2]; for light microscopy tissue is fixed for 2–4 h then transferred to buffer solution until processing; low molecular weight or no extractable nucleic acid
Hollande's	Gastrointestinal and endocrine tissues, small decals, and bones	Picric acid component limits use for molecular testing; low molecular weight or no extractable nucleic acid
Michel transport medium	Renal biopsy transport Cases requiring immunofluorescence	Requires tissue to be washed with PBS prior to processing
Zenker's	Bone marrow biopsies	Poor antigen preservation for IHC; slow penetration; contains mercury; lyses red blood cells; can dissolve iron; silver stains poor after fixation; low molecular weight or no extractable nucleic acid

Table 3
Fixatives for specific disease processes or tissues [2, 8–10]

Type of tissue/diagnosis	Fixative(s)	Special notes
Amyloidosis	Alcohol is preferred, but 10 % neutral buffered formalin (formalin) is acceptable	For special stains, IHC and amino acid sequencing; prolonged storage in formalin causes a gradual decrease in Congo Red staining [1]
Bladder/ureter biopsy	Bouin's or formalin	Superior nuclear detail with Bouin's
Bone marrow aspirate/biopsy	Aspirate—EDTA (*see* **Note 10**) Core biopsy—B-5, formalin, or Zenker's	Decal with EDTA allows for FISH and molecular testing
Breast biopsy	Formalin	Prolonged fixation may result in false negative HER2 staining (*see* **Note 11**) Thick sections do not fix well impeding tissue process and sometimes imperiling histology quality
Fine needle aspiration	95 % alcohol or absolute alcohol	Previously stained smears are an excellent source of material for IHC and molecular testing [10]
GI biopsy	Bouin's, Hollande's, or formalin	
Gouty tophus	Absolute alcohol	Crystals are water soluble and dissolve in formalin
Lymph node	Fresh/fresh frozen—B-5 and formalin	B-5 for 2–3 h then transfer to formalin
Kidney biopsy	1/3 glutaraldehyde for EM 1/3 Zenker's medium, transport medium, or cryopreserved for IF 1/3 formalin for light microscopy	Submit in glutaraldehyde for 2–4 h then transfer to buffer solution
Prostate biopsy	Hollande's or formalin	
Skin biopsy for immunofluorescence	Zenker's or Michel's transport medium	Specimen will need to be washed prior to processing to remove ammonium sulfate
Testes biopsy	Bouin's or formalin	Superior nuclear detail with Bouin's

Table 4
Effect of fixative on nucleic acid preservation

Desirable fixative	Poor fixative
Fixative effect on nucleic acid preservation in paraffin blocks	
10 % buffered formalin (*see* **Note 12**)	B-5
Ethanol	Bouin's
Acetone	Glutaraldehyde
PBS	Carnoy's
Alcoholic formalin	Decalcification solution (acid based)
Fixative effect on nucleic acid preservation in fresh liquid specimen[a]	
EDTA	Heparin

[a]Bone marrow aspirate or Peripheral blood smear (*see* **Note 10**)

4 Notes

1. Tissue fixation continues during processing. If large tissue sections are stuffed into the cassette, not only will thick sections, particularly if they are significantly fatty (e.g., breast tissue), remain poorly fixed throughout processing, but the fluids required to dehydrate and clear the tissue cannot penetrate the tissue. The tissue can potentially be deparaffinized, trimmed, and reprocessed, but at this stage the tissue will be dead and morphology likely not acceptable. As Freida Carson so eloquently puts it, "[t]here is no excuse for overly thick specimens" [17].
2. Tissues incompletely fixed in formalin will continue to undergo fixation when placed in ethanol during processing. The center of the tissue will then show morphologic features of ethanol fixation, possibly altering light microscopic interpretation, but may also result in suboptimal antigenicity in the secondarily fixed areas.
3. Heated fixative solutions are sometimes used to speed up fixation, however care must be taken with tissue greater than 3 mm in thickness as the outside fixes quickly but the center of the tissue is less rapidly penetrated and can be poorly fixed. This can result in a "zonal" fixation effect with differing morphology at the center vs. the periphery of the tissue [3].
4. If a specimen is submitted in saline, discard the saline before adding formalin. Dilution of formalin results in inadequate reagent. In addition, excess blood or other fluids received (i.e., cyst fluid in ovarian specimen) should also be discarded after measurement to avoid dilution. If abundant blood or other fluid is present in the specimen container at receipt, discard and immerse in fresh formalin ("Red/pink formalin = H_2O").
5. Formaldehyde is an immediate irritant to the eyes, upper respiratory tract and skin. If a spill occurs, immediately wash skin

thoroughly. Use a formaldehyde spill kit for small spills of <1,000 mL or call safety services for large spills. Formaldehyde is corrosive and, in the experimental setting, a carcinogen. In humans, exposure to formalin has been associated with slight reduction in memory [18]. Also, if making your own NBF, it should be noted that tap water in some cities introduces AFB-like organisms that can lead to overdiagnosis [19].

6. Gluteraldehyde is easily polymerized and oxidized by air with a resulting drop in pH. This can result in suboptimal tissue fixation and enzyme inhibition. Care must be taken to limit air exposure. Refrigeration is required for storage.
7. Sections will require removal of mercury pigment prior to staining. Tissue must not remain in B-5, or risk becoming overly brittle, and should be transferred to 70 % ethanol [6]. Due to both the health hazards and the precautions required for disposal, many labs no longer use mercury-containing fixatives. Fixatives substituting zinc for mercury are available and are said to offer similar morphology [2].
8. Historically, Zenker's solution was used to fix and decalcify bone marrow biopsies. For reasons similar to those stated in **Note 7**, use of mercury based fixatives is no longer recommended.
9. This is a modification of Bouin's solution; however, the cupric acid stabilizes red blood cell membranes and causes less cell lysis than is seen with Bouin's solution [2].
10. Liquid specimens, including peripheral blood smears and bone marrow aspirates, should be collected in EDTA or acid citrate dextrose tube and stored at room temperature. EDTA chelates ions to inhibit nucleases from degrading nucleic acid. DNA and RNA can be safely extracted with these preservatives up to 48 h at room temperature. Heparin anticoagulant is not recommended because it might interfere with subsequent restriction enzyme or DNA polymerase activity [19].
11. There is some controversy about the reliability of immunohistochemistry results on breast biopsy tissue that has been fixed for greater than 48 h. The current College of American Pathologists recommendations state that formalin fixation over 48 h can result in false negative Her2 staining [6]. A more recent study found only minimal change of semiquantitative H-scoring results of IHC for ER, PR, and Her2 on breast tissue fixed with formalin for 96 h. The authors concluded that results for IHC for breast markers performed on tissue fixed in formalin for up to 96 h should be considered valid [20].
12. There are several non-formalin substitute products currently on the market. While their use is promoted as offering better preservation of nucleic acids, the resulting morphology, and results with immunohistochemistry and FISH, may be suboptimal. For the present the use of 10 % neutral buffered formalin is strongly recommended [21].

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