# FIXATION

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An essential part of all histological and cytological techniques is the preservation of cells and tissues in as reproducible and lifelike manner as possible. To accomplish this, tissue blocks, sections or smears are usually immersed in a fixative fluid, although in the case of smears, merely drying the preparation acts as a form of preservation. The fixatives employed prevent autolysis by inactivating lysosomal enzymes and inhibit the growth of bacteria and molds, which would give rise to putrefactive changes. Furthermore, fixatives stabilize the cells and tissues to protect them from the rigors of subsequent processing and staining techniques.

In performing their protective role, fixatives denature proteins by coagulation, by forming additive compounds or by a combination of the two. Thus, conformational changes in the structure of proteins occur causing inactivation of enzymes. The resulting complexes differ from the undenatured proteins in both chemical and antigenic profiles. The dilemma of fixation has always been that it is necessary to introduce some artifact in order to have a protective effect; by definition, fixatives alter the original chemical composition of the tissues involved.

In addition to altering the chemical nature of the cells and tissues to which they are applied, fixatives also cause physical changes to cellular and extracellular constituents. Viable cells are encased in an impermeable membrane. Fixation breaks down this barrier and allows relatively large molecules to penetrate and escape. Furthermore, the cytoplasm undergoes what is essentially a sol-gel transformation, with the formation of a proteinaceous network sufficiently porous to allow further penetration of large molecules. It must be recognized, however, that different fixatives result in different degrees of porosity; coagulant fixatives, such as B5 and formal sublimate, result in a larger pore size than do non-coagulant fixatives, like formalin. Such changes are obviously of great importance in immunochemistry when it is necessary to demonstrate all but the most superficial antigens in a section or smear.

Most fixative solutions contain chemicals, which stabilize proteins, since this is how protection of the cellular structure is best accomplished. Some fluids have been designed to preserve carbohydrates or lipids. These have not been routinely employed, since histologists and anatomists have mainly required the preservation of microanatomy.

Fixation is always a compromise and the requirements of a fixative vary according to the different techniques employed in visualizing the structure of the cells or tissues. Thus, the techniques for cytology differ completely from those for histology or electron microscopy. Furthermore, the application of different staining methods necessitates other alterations in the fixation protocol, such as air-drying prior to Giemsa staining, and wet-fixation for Papanicolaou's method.

## BLOOD SMEARS AND CYTOCENTRIFUGE PREPARATIONS

Blood smears are preserved by air-drying. The subsequent fixation method used depends upon the staining technique. For routine Romanowsky-type stains, the fixative of choice is a high-grade methanol for 1–3 minutes.

In order to produce the desired morphology with routine blood dyes, it is important that the smear be dry, as is evidenced by the morphology of leucocytes in the thick end of a routine blood film. Fortunately, routine air-drying for 1–2 hours does not have a deleterious effect on most antigens studied immunocytochemically. In fact, it has been shown that many leucocyte surface markers can be preserved for over a week following routine air-drying by storage at room temperature.[1] Thus, it is possible to obtain preparations of these markers of a quality similar to those used for routine morphological evaluation. Using an appropriate chromogen such as diaminobenzidine, it is even possible to counterstain with routine Romanowsky-type methods.

One advantage of air-drying smears is that the cells are more firmly attached to the slide than they are following wet-fixation. This is very important if the slide is to survive the rigors of an immunocytochemical technique. With regard to the choice of fixative, however, methanol is not optimal for all antigens; it is often necessary to fix tissue with anhydrous acetone for 1–3 minutes, especially in the case of the leucocyte surface antigens. Notwithstanding this comment, alternative fixatives are used successfully for these and other antigens. For example, formalin-based fixatives are suitable for use on cytoplasmic antigens and membrane- bound immunoglobulins, while formal-acetone mixtures are employed with certain lymphocytic markers.[2]

Finally, it is noteworthy that air-dried preparations often exhibit relatively weak immunostaining. This is probably because the dried cells exhibit an overall lower antigen density. This can be compensated for by extending antibody and/or chromogen incubation times or by using more sensitive, multiple-step immunocytochemical techniques.

## CYTOLOGY SMEARS

■ Unlike hematologists, most cytologists prefer smears to be wet-fixed immediately after preparation. This preserves the fine structure of the chromatin and helps in the evaluation of nuclear changes. Thus, most cytology smears are immediately fixed in 95% ethanol or are spray-fixed with a carbowax containing alcoholic fluid. Ethanol is satisfactory for preserving many antigens, particularly those used to differentiate melanoma from carcinoma. However, ethanol fixation precludes staining for most leucocyte markers, such as T and B cell antigens. We suggest, therefore, that two preparations be made, one wet-fixed and one air-dried. With wet-fixed smears, one of the main problems is the loss of cells, particularly clumps, during the immunostaining incubations. It is essential to cut incubation times as short as possible by using higher antibody concentrations or by using elevated temperatures.

## CRYOSTAT SECTIONS

■ For immunocytochemistry, cryostat sections give much better antigen preservation than paraffin sections. Additionally, fixative can be used with cryostat sections, allowing the immunochemist to select a different and optimal fixative for each antigen, all from the same block. However, the morphological detail and resolution of the frozen sections is usually considerably inferior to tissue that has been embedded during specimen processing.

Many antigens, such as leucocyte surface markers, survive neither paraffin processing nor fixation with additive fixatives. Accordingly, use either alcohol or acetone. For leucocyte surface antigens, acetone is preferred by most laboratories. Unfortunately, the preservation engendered by acetone is not complete; frozen sections subjected to extended immunochemical procedures often show deleterious morphological changes, including chromatolysis and apparent loss of membranes. Numerous attempts to improve acetone fixation have included the addition of chloroform or dessication, neither of which has proven to be entirely satisfactory.[3] However, thin sections and extended drying prevent the artifacts often seen in frozen-section immunostains of lymphoid tissues fixed in acetone. Extending the drying period to 48 hours will usually result in improved morphology. If it is necessary to stain sections the same day they are cut, sections may be fixed in cold acetone or freshly cut sections may be placed under an infra-red lamp so that the section is heated to 70°C after a 5 second exposure. Slides are then removed, allowed to cool, and the sections are immunostained. This procedure must be carefully controlled to avoid overheating the sections.

Since there are almost as many different procedures as there are laboratories, it is up to the individual technologist and pathologist to determine what sequence of fixation and drying steps will produce the best results for them with the antigens that they are attempting to detect. For further information, see the Tissue Processing chapter for a working procedure.

## PARAFFIN-EMBEDDED SECTIONS

■ By far the largest proportion of samples used for immunostaining is embedded in paraffin, and a number of fixatives have been formulated with this in mind. The most commonly used fixatives are discussed here. There is a plethora of specialty fixatives which will not be covered, but which may appear in literature references given in the bibliography.

■ **FORMALDEHYDE-BASED FIXATIVES** The most popular fixatives contain formalin (40% w/v formaldehyde in water), usually a neutral salt to maintain tonicity and often a buffering system to maintain pH. These fixatives are well tolerated by tissues and have good penetration. This is important since specimens are often large, and fixation may be extended beyond optimal times in routine situations. There may be shrinkage or distortion during fixation or subsequent paraffin-embedding, but generallyformalin-based fixatives are excellent for most immunostains.

Formaldehyde fixes not by coagulation, but by reacting primarily with basic amino acids to form cross-linking “methylene bridges.” This means that there is relatively low permeability to macromolecules and that the structures of intracytoplasmic proteins are not significantly altered. Although many people dislike formalin fixatives, their opinion is often based on studies using suboptimally formalin-fixed tissue. Small (10x10x3 mm) tissue pieces fixed promptly in neutral buffered formalin for 6–24 hours will generally show good cytological preservation and immunolocalization, with a minimum of antigen masking. It is the great variation in time and conditions for fixation that cause the majority of problems in immunochemistry.

Although some antigens are not well demonstrated after fixation in formaldehyde-based fixatives, many can be demonstrated after the use of appropriate pretreatment methods, such as proteolytic enzyme digestion and/or antigen retrieval, particularly if polyclonal antisera are used. If monoclonal antibodies are to be utilized on formalin-fixed, paraffin-embedded tissue sections, there are three considerations, which should be kept in mind:

- Does formaldehyde react with the epitope under investigation?
- Does it react with adjacent amino acids causing conformational changes?
- Does paraffin processing destroy the epitope under investigation?

Monoclonal antibodies are selected for their ability to bind specifically to an epitope on the immunogen. This selection is usually based on immunoenzyme techniques or radioimmunoassays employing the native antigen. If formaldehyde reacts with amino acids within the epitope, the antibody will be unable to bind and therefore will be of no use in formaldehyde-fixed tissue. The same problem may arise with other fixatives and may affect different antibodies.

If there are conformational changes resulting from the reaction of formaldehyde with amino acids adjacent to the epitope, these can often be reversed using proteolytic enzyme digestion or antigen retrieval. If there are conformational changes in the epitope due to tissue processing, these are irreversible. Thus, it is clear that fixatives forming additive compounds may block immunoreactivity, but with the appropriate selection of monoclonal antibodies and the appropriate postfixation treatment, formaldehyde is suitable for monoclonal antibodies against many antigens.

Conformational changes, which destroy epitopes, or alter them to reduce reactivity with the antibody, can occur in a number of ways. The most common alterations occur chemically by fixation, or physically by heat during paraffin-embedding. Many epitopes are sensitive to heat, and during the paraffin-embedding step, tissues are heated to the melting point of wax, usually between 50–60°C. Studies have shown that epitopes of vimentin reacting with some monoclonal antibodies have a half-life of 10–15 minutes at 60°C. Thus, overheating of tissues during embedding or overheating of sections during drying can induce detrimental effects on immunostaining. It is essential not to overheat at any stage of processing if immunostaining is to be optimally sensitive.

When discussing formaldehyde or formalin-based fixatives and comparing the immunocytochemical results obtained in two laboratories, the results often differ. This is hardly surprising since there are many formulas for these fixatives, and even particular batches of formaldehyde may contain different amounts of formic acid and methanol. All of these factors can affect the results of staining fixed tissues. Therefore, although results obtained in different laboratories may be similar, they cannot be expected to be identical.

Given all of these caveats, the following is one standard method of fixation using a neutral buffered formalin solution:

**NEUTRAL BUFFERED FORMALIN SOLUTION (NBF)**

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|---|---|
| Formalin (40% w/v formaldehyde) | 100 mL |
| Sodium phosphate, monobasic, monohydrate | 4 g |
| Sodium phosphate, dibasic, anhydrous | 6.5 g |
| Distilled water | to 1 liter |

This solution is stable for many months at room temperature. Fix small blocks of tissue (10x10x3 mm) for up to 24 hours. Large pieces may be immersed in NBF for a few hours, but blocks should be processed as soon as possible.

Another common fixative is Bouin's solution, which is a mixture of formalin and picric acid. This fixative penetrates rapidly and fixes all tissues very well, except kidney. Fixation time is 1–12 hours depending on tissue thickness. Tissues fixed for longer than 12–24 hours become very brittle. Lipids are reduced in quantity and altered, so lipid-containing antigens may be affected. Tissues fixed in Bouin's solution must be washed in 70% ethanol to precipitate soluble picrates prior to aqueous washes. After cutting sections, the yellow color in the tissue can be removed by treatment with 5% (w/v) sodium thiosulfate, followed by a water wash.

**BOUIN'S SOLUTION**

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|---|---|
| Saturated (1.2% w/v) aqueous picric acid | 75 mL |
| Formalin (40% w/v formaldehyde) | 25 mL |
| Glacial acetic acid | 5 mL |

Saturated picric acid is available commercially so handling of highly explosive picric acid crystals can be avoided. A saturated solution (w/v) contains 1.17 g/100 mL distilled water.

**MERCURIC CHLORIDE-BASED FIXATIVES** In an effort to improve cytological preservation and minimize distortion associated with formaldehyde-based fixatives, as well as to improve on the tinctorial qualities of the stained paraffin sections, mercuric chloride-based fixatives such as formal sublimate and B5 have gained some popularity in histopathology. Like the formalin-based fluids discussed above, these often include a neutral salt to maintain tonicity and may be mixed with other primary fixatives in an attempt to provide a balanced solution. These fluids are generally poor penetrators and are not well tolerated by the tissues. Consequently, small blocks should be employed and the fixation period should be short. Frequently, mercuric chloride-based fixatives are used secondarily to formalin. Tissues are initially fixed in formal saline or neutral buffered formalin, blocks are taken and these are immersed in the mercuric chloride-containing fluid for a further period of fixation.

Mercuric chloride-containing fixatives are additive and coagulative. It is their coagulative properties that cause considerable hardening of the tissue, hence their use originally as "hardening agents." Before the advent of embedding media, it was necessary to immerse tissues in a fluid, which in addition to preventing autolysis and putrefaction, hardened the tissue sufficiently to allow thin slices to be made. These types of fixatives are particularly suitable for the demonstration of intracytoplasmic antigens. Because the permeability of the tissue is greater following coagulant fixatives, antibody penetration is better, resulting in a more intense immunostain. It must be remembered, however, that these fluids are also additive. Therefore, as with formaldehyde-based fixatives, loss of immunoreactivity will occur through blockage of specific epitopes and this

will be particularly evident with monoclonal antibodies. The other major advantage of these coagulant, mercuric chloride-based fixatives, is that the cytological detail is well preserved, improving the localization of the end product of the immunochemical technique, as well as allowing easier morphological interpretation.

B5 is widely advocated for the fixation of lymph node biopsies, both to improve the cytological detail and to enhance the immunoreactivity with anti-immunoglobulin antisera used in phenotyping B cell neoplasms. The immunohistochemical results from such B5-fixed, paraffin-embedded tissue sections are excellent, if only cytoplasmic immunoglobulin staining is required. However, surface membrane immunoglobulin is not stained as readily. Since B5 contains a low percentage of formalin, it may be the formalin that reacts with the surface immunoglobulin. Limited proteolytic enzyme digestion or antigen retrieval will compensate for this, allowing surface immunoglobulin to be demonstrated clearly. However, as a general rule, enzyme pretreatments do not improve, and may actually hinder, immunostaining of tissues fixed in coagulative fixatives.

**B5 STOCK SOLUTION**

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|---|---|
| Mercuric chloride | 2 g |
| Sodium acetate | 2.5 g |
| Distilled water | 200 mL |

**B5 WORKING SOLUTION**

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| B5 Stock solution | 20 mL |
| Formalin (40% w/v formaldehyde) | 2 mL |

Another of the more popular mercuric chloride fixatives is Zenker's solution. Zenker's is subject to the same advantages and disadvantages as B5. Morphological detail is generally well preserved, but penetration is very poor if blocks are more than 3–4 mm thick. For Zenker's, blocks and sections must be cleared of mercury deposits before immunostaining. Fixation time is 2–15 hours at room temperature, depending on tissue thickness. Sections are washed in running water for at least 1 hour to remove potassium dichromate deposits. The tissue is then dehydrated using clearing agent A as the first bath, which helps to remove mercuric deposits prior to sectioning. After cutting sections and collecting on clean glass slides, the tissue is again incubated in clearing solution A for 1–2 minutes. The sections are rinsed in water, and placed in clearing solution for 1–2 minutes.

**ZENKER'S STOCK SOLUTION**

| | |
|---|---|
| Mercuric chloride | 50 g |
| Potassium dichromate | 25 g |
| Sodium sulfate | 10 g |
| Distilled water | to 1 liter |

**ZENKER'S WORKING SOLUTION**

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|---|---|
| Zenker's stock solution | 100 mL |
| Glacial acetic acid | 5 mL |

**CLEARING SOLUTION A**

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|---|---|
| Iodine | 0.5 g |
| 70% ethanol | 100 mL |

**CLEARING SOLUTION B**

Sodium thiosulfate 5% (w/v) in distilled water

■ **ACETIC ACID-ZINC CHLORIDE** This fixative is becoming increasingly popular, partly because it preserves membrane proteins.

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| Zinc chloride | 500 g |
| Formalin (40% w/v formaldehyde) | 3 L |
| Glacial acetic acid | 19 mL |
| Distilled water | 20 L |

■ **PERIODATE-LYSINE-PARAFORMALDEHYDE (PLP)** Numerous compound fixatives have been prescribed for immunochemistry. As with routine histological techniques, these fluids must preserve the microanatomical relationship of cells and cytological details. Periodate-lysine-paraformaldehyde (PLP), first described by McLean and Nakane[4] is thought to be particularly useful since the periodate oxidizes sugars to produce aldehydes which are cross-linked with lysine. The paraformaldehyde stabilizes proteins. A recent modification to this solution involves the addition of potassium dichromate (PLDP).[5] This chemical is added to preserve lipids and the fixative should therefore preserve all protein, carbohydrate and lipid antigenic determinants. It must be noted, however, that since additive compounds are formed, immunoreactivity may be blocked. Furthermore, cytological detail is not as good as with other fluids.

**PLP WORKING SOLUTION**

| | |
|---|---|
| 3% (w/v) Paraformaldehyde | 50 mL |
| M Disodium hydrogen orthophosphate | 100 mL |
| Lysine | 0.9 g |
| Sodium periodate | 0.15 g |

Adjust to pH 7.4.

■ **ETHANOL** Ethanol is not widely employed as a fixative for routine histological techniques due to its poor penetrating ability. However, small pieces of tissue are fixed rapidly and show good cytological preservation. Since alcohols fix through coagulation, without the formation of additive compounds, they are in some ways ideal fixatives for immunocytochemistry. Sainte-Marie first described the use of alcohol fixation and paraffin processing for immunocytochemistry.[6] Alcohol fixation has been widely employed since that time, particularly in research laboratories where the size of specimens and handling requirements are different from the routine histopathology situation.

Since alcohols are coagulant fluids and do not form additive compounds, they permit good antibody penetration and do not block immunoreactive determinants. However, conformational changes can occur. Alcohols precipitate carbohydrates and are therefore particularly useful for surface membrane antigens, which often display carbohydrate-containing epitopes. In this context, they are generally applied to frozen sections or smears. Proteolytic digestion or antigen retrieval is of no use following alcohol fixation and results in destruction of the tissue section or smear.

■ **ACETONE** Acetone is an excellent preservative of immunoreactive sites, leaving most sites intact, but it is a very poor penetrator. For this reason, it is used only for smears and cryostat sections. Fixation is not complete, and extended incubation in buffers may result in chromatolysis and loss of membranes. Refer to sections on blood smears and cryostat sections for procedures.

## FIXATION FOR IMMUNOELECTRONMICROSCOPY

■ The dilemma of trying to obtain good morphological preservation while maintaining immunoreactivity is even more apparent in ultrastructural immunochemical studies. For routine electronmicroscopy, it is usual to employ glutaraldehyde primary fixation followed by postfixation in osmium tetroxide. This combination produces excellent ultrastructural detail with good preservation of membranes. With immunochemistry, however, the combination of glutaraldehyde and osmium tetroxide is not generally useful. In some instances, it is possible to pretreat ultrathin sections with hydrogen peroxide or sodium metaperiodate to counteract the deleterious effects of osmium. Unfortunately, this is not possible in all cases. Furthermore, the glutaraldehyde primary fixation is not suitable for many antigens, at least when employing the concentrations of glutaraldehyde used for routine electronmicroscopy. Consequently, for immunoelectron microscopy studies, paraformaldehyde is often employed either alone or in mixtures containing very low (0.1–0.2%) concentrations of glutaraldehyde. Either perfuse the animal with fixative after flushing with saline, or immerse small (1x1x1 mm) pieces for 2 hours at room temperature. Do not postfix in osmium. Process routinely to Epon, Lowicryl, LR white or LR gold.

**PARAFORMALDEHYDE-GLUTARALDEHYDE WORKING SOLUTION**

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|---|---|
| 0.2 M Phosphate buffer, pH 7.4 | 50 mL |
| 8% (w/v) Paraformaldehyde | 25 mL |
| 25% (w/v) Glutaraldehyde | 0.8 mL |
| Distilled water | 24.2 mL |

For a more detailed discussion on fixation in electron immunocytochemistry, refer to the texts listed in the Bibliography.

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