CHAPTER 8

Overview of Cell Fixation and Permeabilization

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1. Introduction

The localization of proteins and carbohydrates within cells and tissues with specific antibodies has long been proven to be a valuable technique. Immunolocalization procedures allow one not only to detect well-characterized cellular structures but also to provide information about newly characterized proteins and carbohydrates. This chapter will review some of the advantages and drawbacks of common chemical fixation and permeabilization methods used for immunolocalization at the level of light microscopy.

It is usually impossible to perform immunohistochemical microscopic studies with living specimens. Antibodies are large molecules that need to be microinjected into living cells if internal structures or proteins are to be localized. Microinjection, however, is not practical if the antigen to be localized resides within an organelle. In the case of surface antigens, antibody solutions can be applied to the outer surface of living cells, but long incubation with antibody solutions can result in the internalization of antibody–protein complexes through endocytosis. Antibodies can also nonspecifically bind to cell surfaces by their F_c regions and lead to false results *(1)*. Specific proteins can be studied with the technique of fluorescent analog cytochemistry, which allows fluorochrome-derivitized proteins to be functionally traced in living cells *(2)*. Although a powerful technique, it usually has limited applications, and is often not practical for most laboratories or routine procedures *(1)*. Large amounts of protein

From *Methods in Molecular Biology, Vol. 34: Immunocytochemical Methods and Protocols*
Edited by L C Javois Copyright © 1994 Humana Press Inc , Totowa, NJ

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need to be isolated and derivitized with a fluorochrome in a way that does not disturb its functional properties *(2)*. Therefore, most laboratories rely on chemical fixation and permeabilization of cells before treatment with antibody solutions to determine the location(s) of antigens within tissues and cells *(3)*.

It is helpful to know the chemistry of fixatives in order to understand their action and avoid artifacts *(4)*. Most commonly studied antigens are either proteins or carbohydrates. Many of these molecules are soluble in aqueous solutions and need to be fixed in place in cells. Insoluble antigens also need to be structurally preserved *(1)*. All chemical fixatives will cause chemical and conformational changes in the protein structure of cells with lesser changes noted for carbohydrate antigens *(5)*. Secondary and tertiary structures in proteins are the most important for eliciting antigenicity, and chemical fixatives usually disturb these structures *(3)*.

Under ideal conditions, fixation of tissues and cells should minimally change cellular structure and chemical composition *(4)*. For immunohistochemical analysis, the fixative should preserve cellular structure as well as prevent the loss and/or migration of antigens. Unfortunately, those fixation methods that minimally affect antigenic epitopes are generally not the best for preserving morphology. Conversely, the methods that best preserve morphology are those most disruptive to antigenic sites *(4)*. The loss of antigenicity increases with the fixative concentration and the time of fixation *(3)*. One should therefore realize that there is no "perfect" fixative and strike a compromise between preserving cellular structure and maintaining the antigenicity of the epitopes of interest *(3)*.

There are two basic types of fixatives: coagulants and crosslinking agents. Although coagulant agents tend to induce artifacts (extraction, tissue shrinkage, granular/reticular cytoplasm), they have been found to be effective in light microscopy particularly for large-molecular-weight antigens and polymerized structural proteins *(1,3,4,5)*. Because crosslinking fixatives act by forming chemical bonds, severe conformational changes of proteins can occur owing to the modification of reactive groups *(1)*. Crosslinking fixatives can also cause artifacts either by linking low-molecular-weight antigens to larger structural proteins or by causing steric blockage of antibody access to the antigenic epitopes *(1)*. Protein and carbohydrate antigens can also be lost through extraction during fixation procedures because of the solubility of most antigens in aqueous solutions *(6)*.

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Antibodies are large molecules. Immunoglobulin G molecules have an arm-to-arm distance of 146 Å *(7)*, and F_{ab} fragments have dimensions of 30 × 40 × 50 Å *(8)*. Molecules of this size cannot diffuse into and out of cells. Fixatives, particularly crosslinking fixatives, act upon membrane proteins and reduce the overall membrane permeability *(3,9)*. Therefore, one needs to "open" plasma membranes and organelle membranes in order to allow antibody access to intracellular and intraorganellar antigens. Solvents, saponins, and nonionic detergents are the most commonly used reagents for membrane permeabilization. It should be noted, however, that plasma membranes and some organelle membranes show different properties and, therefore, may need different conditions to affect permeabilization *(10)*.

2. Commonly Used Fixatives

2.1. Solvents

Solvents such as alcohols and acetone are strong coagulant fixatives. They act by displacing water, breaking hydrogen bonds, and thus disrupting the tertiary structure of proteins *(11)*. With this procedure soluble proteins are precipitated *(11)*, but neither carbohydrates nor nucleic acids are fixed in place and are removed by washing *(5)*. Lipids in both the membranes and cytoplasm are solubilized and extracted *(5)*. The displacement of water causes cellular shrinkage and destroys most organelles within the cell *(5,11)*. At low temperatures (0 to ⁻20°C), ethanol precipitates proteins without denaturing them and has been found to be practical for fixing large-molecular-weight antigens, such as assembled cytoskeletal proteins *(1,11,12)*. Low-molecular-weight antigens (<100 kDa) are generally extracted *(1,6)*. These fixatives, because of their extreme disruptive effects on cellular organelles, are useful only for light microscopy and not electron microscopy *(1)*.

2.2. Formaldehyde/Paraformaldehyde

Formaldehyde is a colorless gas that is soluble in water *(3)*. Commercial aqueous preparations of formalin contain 37–40% (w/w) solubilized gas. They also contain formic acid (<0.05%) and 10–15% methanol which is added to prevent the polymerization of formaldehyde into paraformaldehyde *(3,11)*. Methanol and formic acid make these solutions an unacceptable fixative for fine structures *(9)*. Paraformaldehyde is a polymerized form of formaldehyde that dissociates at 60°C and neu-

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tral pH. Freshly prepared solutions of paraformaldehyde are preferred for most immunochemical procedures, because it provides a fixative free of extraneous additives and is usually the conservative fixative of choice when beginning the development of a fixation procedure *(3,5)*.

Although it is the simplest aldehyde, the chemistry of formaldehyde reactions with proteins is quite complex *(11)*. Formaldehyde crosslinks proteins by addition to amino, amido, guanidino, thiol, phenolic, imidazolyl, and indolyl groups, and forms hemiacetal derivatives *(3)*. If the hemiactetal addition products are in close proximity to other proteins, they react by condensation to form chemically stable methylene bridges that crosslink the proteins *(11)*. Formaldehyde addition reactions are readily reversible by washing with water or alcohol *(5,11)*. Prolonged washing of tissues can, in some cases, restore antigenicity to fixed proteins *(1)*. The maximum levels of protein crosslinkages occur in the pH range of 7.5–8.0 *(3)*. At lower pH, primary amino groups are unreactive *(1)* and thus, crosslinking reactions are not favored. Addition of bicarbonate to formaldehyde is reported to minimize extraction of proteins from tissues presumably by raising intracellular pH and increasing protein crosslinkages *(3)*.

Formaldehyde prevents the extraction of glycogen, but does not preserve soluble polysaccharides. Acid mucopolysaccharides are also not preserved unless they are bound to proteins *(3)*. Formaldehyde is a good fixative for lipids particularly if 1–2 m*M* Ca^{2+} or Mg^{2+} is included in the fixative vehicle *(4,5,11)*. Membrane fixation is improved by reducing lipid extraction *(4)*. It is thought that fixation with formaldehyde lowers the solubility of membrane phospholipids in water *(11)*.

2.3. Glutaraldehyde

Glutaraldehyde, glutaric acid dialdehyde, is a fixative that is very effective in preserving fine structure *(3)*. Glutaraldehyde fixation is usually paired with osmium tetroxide postfixation to provide excellent cytological preservation for electron microscopy. The postfixation steps, however, severely lower the antigenicity of proteins for immunodetection either through protein cleavage, oxidation, or conformational changes induced by osmium tetroxide *(1,9)*. Fixation with glutaraldehyde alone also results in lowered protein antigenicity *(3,9)*.

Because it is a dialdehyde, glutaraldehyde acts to crosslink proteins by means of its two aldehyde groups. The chemical reactions of glutaralde-

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hyde with proteins are not well understood and the topic of much debate. Current opinion seems to hold that glutaraldehyde is most reactive with the ε-amino groups of lysine *(3)*. Glutaraldehyde acts rapidly to crosslink proteins and, thus, renders them insoluble *(3)*. Intramolecular crosslinkages tend to predominate over intermolecular bonding, and major conformational changes occur because of the disruption α-helical structures. These types of protein shape changes can lead to lowered immunoreactivity by blocking or masking reactive epitopes *(3,9)*. The reactions of glutaraldehyde with carbohydrates are also not well understood. The fixative most likely reacts with polyhydroxyl compounds to form polymers in mucopolysaccharides *(3)*. Fixation with glutaraldehyde is essentially irreversible *(1)*.

Highly purified solutions of glutaraldehyde are used by enzyme cytochemists, but seem to be seldom used by immunocytochemists *(1)*. Impurities in glutaraldehyde, cyanide and arsenic in some commercial preparations, can greatly contribute to reducing protein antigenicity *(13)*. "Undefined" impurities that absorb at 235 nm can form on prolonged exposure to air *(4)*. The purity of glutaraldehyde solutions can be checked by determining the $A_{235/280}$ ratio of the solution. A value of >0.2 is generally associated with impure solutions. Therefore, fresh solutions of glutaraldehyde prepared from stocks packaged under inert gas are usually the best. Charcoal absorption or distillation of the solutions is also an option for purification *(3,4)*.

Incomplete fixation with glutaraldehyde can cause serious artifacts in immunocytochemistry because of unreacted free aldehyde groups. These reactive groups, if left unchanged, can react with antibodies and nonspecifically link them to proteins. The free aldehyde groups must be irreversibly blocked either through reduction with sodium borohydride *(14)* or by blocking with phenylhydrazine *(11)*, ethanolamine, or lysine *(1)*. Unreacted free aldehyde groups of the fixative can also cause high background autofluorescence in fluorescence procedures *(14)*.

2.4. Other Chemical Fixatives

Picric acid (trinitrophenol) and trinitroresorcinol when added to fixative solutions give greater fine structural preservation of cells *(11,12)*. These compounds cause coagulation of proteins by forming salts with positively charged groups of proteins *(11)*. The protein precipitates formed retain their antigenicity *(3)*. Picric acid or trinitroresorcinol is

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most often added to formaldehyde solutions, and fixative solutions of glutaraldehyde that contain these compounds have been shown to be effective for immunoelectron microscopic techniques *(13)*. These fixatives work particularly well to preserve membrane structure *(4)*.

Carbodiimide crosslinking fixatives are beginning to be tested as fixative agents. These compounds act by crosslinking carboxyl groups to amine groups through amide bond linkages *(1)*. The carboxyl and amine reactive groups must be in close proximity, however, for crosslinkage to occur *(15)*. They have been found to be inferior to aldehydes in preserving cellular structure, particularly for electron microscopic procedures. They may, however, preserve some antigenic sites that are destroyed by aldehyde fixatives *(1,15)*. Carbodiimides show promise for fluorescence techniques in that they do not cause significant background fluorescence *(15)*.

3. Commonly Used Permeabilization Agents

3.1. Solvents

Alcohols and acetone are the simplest kinds of membrane permeabilization agents. They act by dissolving membrane lipids and, thus, rendering the membrane permeable to antibodies *(5)*. Because of their coagulant effects on proteins, these solvents can be used as a "one-step" fixative and permeant *(16)*.

3.2. Saponins and Lysolecithin

Saponins are natural compounds derived from plants. Saponins are generally the best permeant choice for routine cytoplasmic antigen localizations *(10)*. They act on membranes by interacting with cholesterol, plant sterols, phospholipids, and proteins. Saponin treatment is thought to break the associations between cholesterol and phospholipids causing the formation of 120–150 Å membrane openings, resulting in only small losses of cholesterol *(17)*. Some of these membrane openings are transient in nature and some permanent with most being formed 10–20 s after treatment with the agent *(17)*. Because of the transient nature of some of these membrane openings, it is usually recommended that saponin be included in all solutions throughout antibody treatments.

Lysolecithins act by dissolving cholesterol and cause massive losses of the sterol from membranes *(17)*. Lysolecithins have been shown to cause the formation of openings 300–400 Å in diameter in erythrocyte plasma membranes *(18)*. Unlike saponins, lysolecithin membrane openings are permanent.

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3.3. Nonionic Detergents

Polyoxyethylene nonionic detergents (Triton X-100, Nonidet P-40, Tween-20, Brij 35, and so forth) are used most often in immunochemical techniques because they generally do not denature proteins. Detergents act by intercalating into phospholipid bilayers, solubilizing lipids and integral membrane proteins, and thereby disrupting the membrane *(19)*. Hydrophobic proteins become enveloped in detergent and are easily washed away. For some antigens, particularly those localized within mitochondria or the nucleus, nonionic detergent treatments are required because the membranes of these organelles do not contain large amounts of cholesterol and are not rendered permeable by saponins *(10)*.

Treatment of cells with nonionic detergents, while considered mild, is not without hazards; it can never be assumed that a detergent will not affect protein structure. Detergents with long hydrocarbon chains can denature some proteins *(19)*. High levels of oxidizing impurities capable of reacting with sulfhydryl groups in proteins have been found in commercial preparations of Triton X-100 and Brij 35 *(20)*. These oxidizing compounds can lead to loss of antigenicity and to high background autofluorescence in some fluorescence procedures. Some integral membrane proteins may be removed from the plasma membrane and organelle membranes by detergent treatments even after the cells have been fixed *(10)*. Such extraction of hydrophobic proteins can artifactually suggest lack of reactivity with antibodies.

4. Special Considerations For Plant Cells

The presence of a cellulosic cell wall and vacuoles constitutes the major difference between plant and animal cells. These two structures allow the plant cell to maintain a high internal turgor pressure, a factor that needs to be considered when choosing an osmotically compatible fixative vehicle. Fixative solutions, nevertheless, are generally the same for plant and animal tissues *(3)*. The cytoplasm of animal cells has a higher protein concentration per unit volume than plant cells and, thus, crosslinking fixatives give good results with animal cells *(3)*. Meristematic plant cells with dense cytoplasm and small vacuoles show better fixation with these agents than mature cells *(3)*.

Plant plasma membranes and tonoplasts (vacuole membranes) are particularly sensitive to fixation conditions and fixation of the vacuoles can be quite problematic. If the fixation is inadequate, then the tono-

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plast can rupture and release hydrolytic enzymes into the cytoplasm *(3)*. Often fixatives at higher concentrations are used for plant cells in order to compensate for dilution by the vacuolar contents. The air in intercellular spaces can also hinder the penetration of fixatives into plant tissues. However, putting the tissue under a vacuum during fixation assists fixative penetration. It is usually best to apply a slight vacuum since stronger vacuums have been found to cause structural damage most often resulting in separation of the plasma membrane from the cell wall *(3)*.

Although some report that antibodies are able to penetrate cell walls fully, immunofluorescence micrographs of cell-wall protein localizations suggest that this is not the case *(21)*. Antibody solutions infiltrated under vacuum into stem tissues do not appear to reach inner surfaces of the cell walls, whereas antibodies applied to cut surfaces of the stem clearly do (*see* Fig. 3 in ref. *21*). In order to facilitate the penetration of antibodies into plant cells, the cell walls need either to be "opened" or removed. This is most often accomplished by digestion with the enzymes cellulase and/or pectinase *(22)*.

5. Choosing A Fixation/Permeabilization Protocol

Many factors need to be considered when choosing and/or developing a fixation protocol for antigen localization. The fixative regime will depend in large part on the antigen being studied *(1)*. Some fixation methods can be epitope-specific in that a particular antigen may or may not react with different antibodies *(1)*. This can be either a disadvantage or an advantage in that: (1) the same tissue may not react with different antibodies or (2) one can test different stocks of antibodies prepared against a particular antigen in order to find one that reacts with tissues fixed by a "preferred" method. The choice of fixative vehicle is important because of its osmotic and ionic effects on cellular structures particularly organelles *(9)*. Extraction of soluble proteins has been found to be a particular problem with hyperosmolar fixatives, and isotonic or hypotonic solutions are generally preferred *(1)*. Fixative vehicles, as with the fixatives themselves, usually need to be adjusted for the particular cell type or tissue under investigation *(9)*.

Melan and Sluder *(6)* showed that localizations of soluble proteins in cells can differ markedly from in vivo distributions depending on the fixation/permeabilization regime chosen. Figure 1 (reproduced from *[6]*) shows an array of artifactual localizations obtained with soluble fluo-

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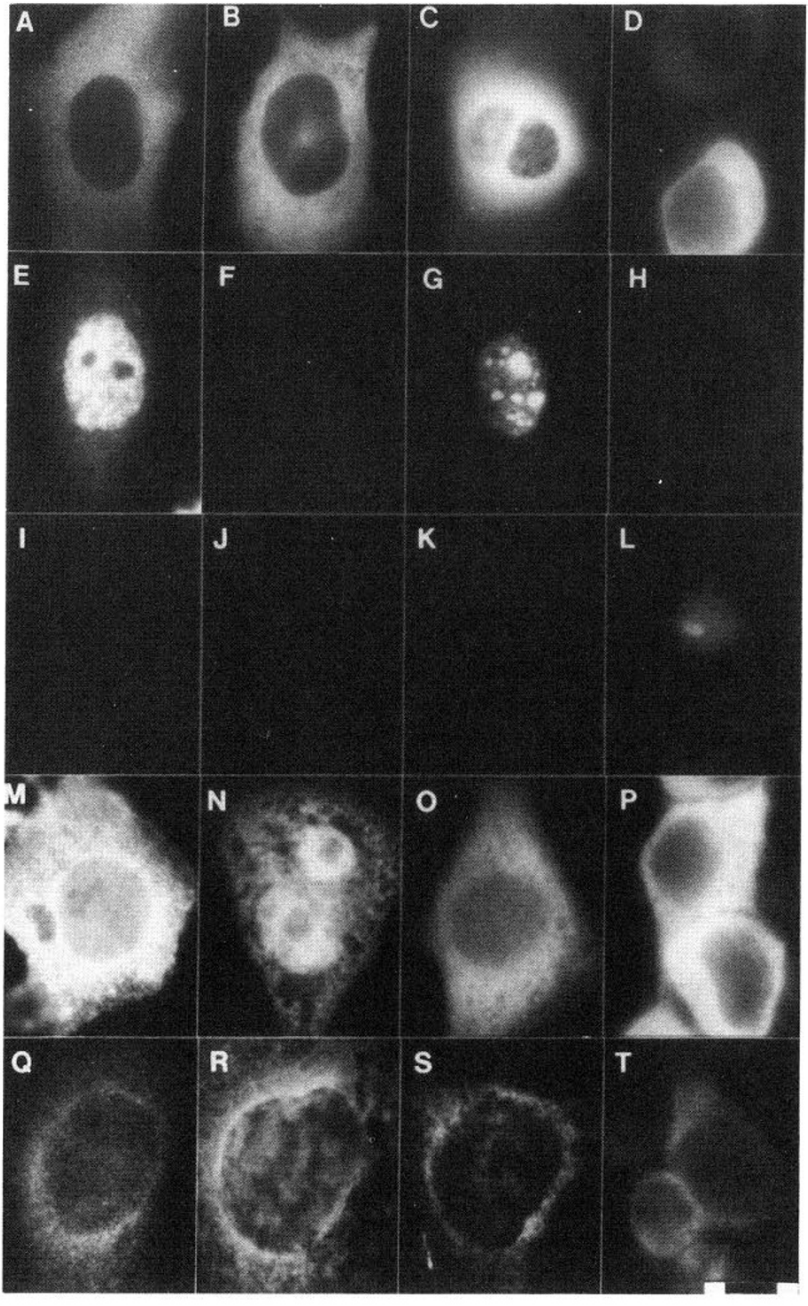

Fig. 1 (*see* caption on p. 64).

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rescein-labeled bovine serum albumin (FITC-BSA) in various fibroblast cell lines. Although the FITC-BSA was evenly distributed in the cytoplasm and excluded from the nuclei in living cells (A–D), different fixation and permeabilization regimes led to striking relocation and extraction artifacts. A low level of global redistribution of extracted soluble proteins to all cells, presumably by transfer through washing solutions, was also noted *(6)*.

This study cautions that unless a cellular structure is thoroughly known, immunolocalizations in that structure may be suspect *(4,6)*. Often the intracellular distribution of an antigen is not known beforehand, and these types of artifacts can lead to uncertainty in the results of immunolocalizations. It is recommended that the location of antigens be determined by several methods (i.e., various fixatives and permeabilization agents) before strong conclusions are drawn. Optimal fixation, therefore, requires a systematic evaluation of reagents and procedures *(3)*. It is generally best to begin with an established procedure and then modify the procedure as needed while keeping in mind the potential for artifactual localizations *(3)*.

Fig. 1 (appears on p. 63). Distribution of FITC-conjugated BSA in various fibroblast cell lines under different fixation/permeabilization regimes. (**A–D**) Protein distribution in living cells—PtK_1, CHO, 3T3, and HeLa cells, respectively. The protein is excluded from the nuclei of all cells. (**E–H**) Protein distribution in cells extracted for 10 min with 0.1% Triton X-100 before fixation for 30 min with 3.7% formaldehyde—PtK_1, CHO, 3T3, and HeLa cells, respectively. Nuclear fluorescence is seen in PtK_1 (E) and 3T3 (G) cells. (**I–L**) Protein distribution in cells extracted for 10 min with 1% Triton X-100 before fixation for 30 min with 3.7% formaldehyde—PtK_1, CHO, 3T3, and HeLa cells, respectively. No fluorescence is detected in the cells with the exception of some nuclear fluorescence seen in HeLa cells (L). (**M–P**) Protein distribution in cells fixed for 30 min with 3.7% paraformaldehyde before permeabilization for 10 min with 0.1% Triton X-100. Fluorescence is seen primarily in the cytoplasm with the exception that nuclear fluorescence is seen in PtK_1 (M) and CHO (N) cells. (**Q–T**) Protein distributions in cells fixed for 5 min with 90% methanol, 50 m*M* EGTA at –20°C—PtK_1, CHO, 3T3, and HeLa cells, respectively. All cells show an overall low fluorescence, fibrous-textured cytoplasmic fluorescence and bright staining at the periphery of the nucleus, 10 μm per scale division (black bar). Reproduced with permission from ref. *6*.

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Acknowledgments

I thank Greenfield Sluder and Dean P. Whittier for critical evaluation and helpful comments on the manuscript. I also thank Frederick J. Miller and Michael Rodriguez for photographic assistance.

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