

Chapter 8

Cell Fixatives for Immunostaining

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Abstract

Fixation is one of the most critical steps in immunostaining. The object of fixation is to achieve good morphological preservation, while at the same time preserving antigenicity. Tissue blocks, sections, cell cultures or smears are usually immersed in a fixative solution, while in other situations, whole body perfusion of experimental animals is preferable. Fixation can be accomplished by either chemical or physical methods. The chemical methods include cross-linking agents such as formaldehyde, glutaraldehyde and succinimide esters as well as solvents such as acetone and methanol, which precipitate proteins. Of the physical methods, freezing tissue and air drying are most widely used. This chapter deals with the chemical fixation methods most commonly used for light microscopy.

Key words: Fixation, Formaldehyde, Glutaraldehyde, Methanol, Acetone, PLP, Methacarn, Carnoy's, Bouin's

1. Introduction

Fixation is one of the most critical steps in immunostaining. The object is to achieve good morphological preservation while at the same time preserving antigenicity (1, 2). This becomes more problematic when one is dealing with intracellular antigens and the sample must be permeabilized to allow access to the antigen. The goal of fixation is to preserve cells and tissues in a life-like manner. Tissue blocks, sections, cell cultures or smears are usually immersed in a fixative solution (3). In some cases, perfusion of the whole animal via its circulatory system with fixative solutions is preferred. Fixation is also necessary to stabilize the sample and protect it from the deleterious effects of the immunostaining process. Fixation also helps to prevent artifactual diffusion of cell components, arrests enzymatic activity,

and avoids decomposition of the tissue. Furthermore, the process of fixation helps to preserve the sample by inhibiting bacterial and fungal growth and by inhibiting lysosomal enzymes whose activity could lead to autolysis. Tissues vary in their composition, having different protein and lipid content. They also differ in the structural arrangement of these components. The fixatives, formaldehyde and glutaraldehyde, which are best for the preservation of tissue and protein structure (4) do so by cross-linking proteins making access of reagents difficult and possibly masking some epitopes. There is no universal fixative and with some antigens it may be necessary to try multiple fixatives. Any given fixative may preserve the immunoreactivity of one epitope, but it may destroy other epitopes on the same antigen (1). Fixation can be accomplished by either chemical or physical methods. The chemical methods include cross-linking agents such as formaldehyde, glutaraldehyde and succinimide esters as well as solvents such as acetone and methanol, which precipitate proteins (5). A new proprietary fixative, HOPE (Hepes-glutamic acid buffer-mediated Organic solvent Protection Effect), that uses no cross-linking agents has recently been developed (6). The physical methods most commonly used are freezing (*see* Chapter 10) and air drying. The most widely used fixative for immunostaining is formaldehyde (*see* Note 1), either alone or in combination with a solvent such as methanol or another substance such as picric acid (trinitrophenol) that coagulates proteins. Formaldehyde is a pungent, colorless, toxic, water-soluble gas that rapidly polymerizes into paraformaldehyde. Methanol is often added to commercial preparations to stabilize the formaldehyde (*see* Note 2). Most of the formaldehyde in a freshly prepared aqueous solution is in the form of methylene glycol, a small molecule that penetrates the tissue rapidly. Formaldehyde-based fixatives stabilize tissues by reacting primarily with basic amino acids to form methylene cross-links. The number of methylene bridges formed depends on the concentration of formaldehyde, temperature, pH and time of exposure (7). These fixatives are relatively gentle and are partially reversible. Formaldehyde is usually used as a 2–10% solution in buffer, such as phosphate-buffered saline, or in cold methanol. It is also a component of Bouin's fixative. This chapter deals primarily with fixatives most commonly used for light microscopy. However, for electron microscopy, formaldehyde is frequently used in combination with low concentrations of glutaraldehyde (*see* Note 3 and Chapter 37). Of the physical methods, freezing tissue is most widely used (*see* Chapter 10). Tissue blocks may be fixed before freezing or unfixed frozen sections may be fixed *in situ*.

2. Materials

1. 8% Paraformaldehyde Stock (*see* Note 4): Heat 800 mL of distilled H_2O to 55°C . Add 80 g of paraformaldehyde prill type and stir continuously on heat/stir plate. Do not allow powder to settle. Do not allow temp to exceed 60°C (*see* Note 5). Stir solution for 15 min at 55°C . Add a few drops of 1 N NaOH until the solution just clears. A small amount of flocculent material may remain. Filter through Whatman No. 1 filter paper. Bring volume to 1 L and store at 4°C for up to 1 month.
2. Lysine Stock: Dissolve 16.4 g of L-Lysine in 300 mL distilled H_2O . Adjust pH to 7.4 with 0.1 M Na_2HPO_4 . Bring volume to 450 mL with distilled H_2O . Bring volume to 900 mL with 0.1 M Sorenson's Phosphate buffer. This may be stored at 4°C for up to 3 weeks.
3. 0.4 M Sorenson's phosphate buffer Stock: Dissolve 7.176 g sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) in 100 mL distilled H_2O . Dissolve 49.4 g of sodium phosphate (NaPO_4) in 750 mL distilled H_2O . Mix the sodium phosphate monobasic monohydrate and sodium phosphate solutions in a 1 L cylinder. Bring volume to 1 L with distilled water. The pH should be 7.6. Do not adjust pH. This may be stored at room temp for up to 6 months. For use dilute with distilled water to 0.1 M.
4. Paraformaldehyde/Lysine/Periodate Fixative (PLP) (2) (*see* Note 6): Place 30 mL of lysine stock in a 50 mL screw cap tube. Add 10 mL 8% paraformaldehyde. Add 0.085 g of Sodium *m*-periodate powder. Shake until powder dissolves.
5. Phosphate Buffered Saline (PBS): 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 , 2.16 g Na_2HPO_4 bring to 1 L with distilled water. pH should be 7.4.
6. Methacarn Methanol Carnoy's fixative: Freshly prepare, by volume, a solution of 60% methanol, 30% chloroform and 10% glacial acetic acid.
7. Glutaraldehyde should be purchased as a 70% solution of purified glutaraldehyde packaged in glass ampules under nitrogen.
8. Bouin's Fixative (*see* Note 7): Mix together 300 mL saturated picric acid (*see* Note 8), 100 mL commercial formalin (*see* Note 9) and 20 mL glacial acetic acid.

3. Methods

3.1. Paraformaldehyde/ Lysine/Periodate

1. Place sample in PLP fixative for a maximum of 24 h.
2. Rinse sample with 0.1 M Sorrensons phosphate buffer.
3. Process for immunomicroscopy.

3.2. Formaldehyde in PBS

1. Dilute formaldehyde (*see* Note 10) to 1–4% in PBS.
2. Immerse sample in formaldehyde solution for 15 min to 12 h at room temperature depending on the specimen.
3. Rinse well, a minimum of three times, in PBS.
4. Process as desired.

3.3. 2% Formaldehyde in Methanol (*see* Note 11)

1. Dilute freshly prepared formaldehyde stock or commercial formaldehyde to 2% in absolute methanol at -20°C (*see* Note 12).
2. Place sample on ice (*see* Note 13).
3. Add fixative and fix for 2–5 min on ice.
4. After 2–5 min, dilute the methanol-formaldehyde with cold PBS (*see* Note 14).
5. Remove half the solution.
6. Replace with cold PBS.
7. Repeat two more times.
8. Rinse in cold PBS.
9. Process as usual.

3.4. Cold Methanol or Acetone (*see* Note 11)

1. Place sample on ice (*see* Note 13).
2. Add absolute methanol or acetone at -20°C (*see* Note 12) and fix for 2–5 min on ice.
3. After 2–5 min, dilute the methanol or acetone with cold PBS (*see* Note 14).
4. Remove half the solution.
5. Replace with cold PBS.
6. Repeat two more times.
7. Rinse in cold PBS.
8. Process as usual.

3.5. Methacarn (Methanol Carnoy's fixative) (*see* Note 15)

1. Fix thin slices or small blocks of tissue for 6–8 h at room temperature in Methacarn.
2. Rinse in two changes of methanol, 1 h each.
3. Rinse in several changes of methyl benzoate.
4. Clear, infiltrate and embed as usual.

3.6. Glutaraldehyde–Formaldehyde (see Note 16)

1. Dilute stock glutaraldehyde solution to 0.5% in PBS containing 2% formaldehyde (*see* Note 17).
2. Fix samples for 20 min to 1 h in fixative.
3. Rinse three times in PBS.
4. Process as usual.

3.7. Bouin's Fixative (see Note 18)

1. Fix tissue by immersion for 1–12 h depending on sample size (*see* Notes 19 and 20).
2. Wash in 70% alcohol to precipitate soluble picrates.
3. Wash in water.
4. Dehydrate and embed tissue in paraffin.
5. Cut sections may be treated with 5% aqueous sodium thiosulfate followed by washing in water to remove any residual picric acid.

4. Notes

1. Commercial formalin preparations should not be used. Formalin is an aqueous solution of formaldehyde that typically contains 37–40% formaldehyde, 10–15% methanol and <0.05% formic acid and usually contains impurities. It has a high background fluorescence.
2. For immunostaining formaldehyde should be freshly prepared from paraformaldehyde powder or purchased as an EM grade aqueous paraformaldehyde solution sealed in ampoules under nitrogen.
3. Since glutaraldehyde is autofluorescent, it should not be used as a fixative for immunofluorescence.
4. *Paraformaldehyde is toxic. Prepare in fume hood.*
5. If the temperature exceeds 60° C, formic acid will form.
6. PLP fixative should be made just before use.
7. Bouin's fixative is *carcinogenic, irritant and toxic*. If you do not use it routinely, it is better to buy small amounts of the fixative ready-made than to make it.
8. To make a saturated solution of picric acid, add distilled water to the manufacturer's container and let it stand in a dark place. Do not let the picric acid dry out as it can become explosive.
9. Commercially available formalin is 37–40% formaldehyde (*see* Note 1) and may destroy many epitopes.

10. Formaldehyde should either be freshly prepared from paraformaldehyde or purchased as a purified solution (*see* Note 2).
11. This method is better suited to cultured cells grown on coverslips or to frozen section on glass slides. It is not well suited to blocks of tissue.
12. Absolute methanol or acetone may be stored in the freezer at -20°C until just before use.
13. Sample should be transferred to glass containers before fixation.
14. PBS should be added to the solvent to prevent evaporation of methanol or acetone and drying of the sample.
15. Methacarn is a fixative used on tissues to be paraffin-embedded and immunochemically stained in which methanol has been substituted for the ethanol in Carnoy's fluid. This methanol–Carnoy mixture is referred to as methacarn solution. It preserves certain tissue and cell components such as collagen and cytoskeletal elements exceptionally well for immunoperoxidase and other immunochemical staining (8).
16. Glutaraldehyde crosslinks proteins and may alter protein configuration and mask epitopes. Because of its cross-linking properties, penetration of reagents may be impaired. For immunocytochemistry, it is usually used in low concentration in combination with formaldehyde.
17. The concentration of glutaraldehyde may vary between 0.25 and 1% and the concentration of formaldehyde may vary between 2 and 4%.
18. Work in a well-ventilated area, wear gloves, lab coat and goggles. Avoid contact and inhalation. Toxic through skin exposure.
19. Tissues fixed for longer than 12–24 h become very brittle.
20. Lipids may be extracted or altered, so lipid-containing antigens may be affected.

References

1. Larsson L-I (2000) Immunocytochemistry: theory and practice. CRC Press, Boca Raton, FL
2. McLean IW, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative A new fixative for immunoelectron microscopy. *J Histochem Cytochem* 22:1077–1083
3. Presnell JK, Schreibman MP (1997) Humanson's animal tissue techniques. The Johns Hopkins University Press, Baltimore and London
4. Mason JT, O'Leary TJ (1991) Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation. *J Histochem Cytochem* 39:225–229
5. Kiernan JA (1981) Histological and histochemical methods: theory and practice. Pergamon, Oxford, UK
6. Olert J, Wiedorn KH, Goldmann T, Kuhl H, Mehraein Y, Scherthan H, Niketeghad F,

- Vollmer E, Muller AM, Muller-Navia J (2001) HOPE fixation: a novel fixing method and paraffin-embedding technique for human soft tissues. *Pathol Res Pract* 197:823–826
7. Werner M, Chott A, Fabiano A, Battifora H (2000) Effect of formalin tissue fixation and processing on immunohistochemistry. *Am J Surg Path* 24:1016–1019
8. Holde P, Waldrop FS, Meloan SN, Terry MS, Conner HM (1970) Methacarn (methanol–Carnoy) fixation. *Histochem Cell Biol* 21:97–116