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Models of Fixation and Tissue Processing

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Abstract

Fixation and processing of tissue to paraffin blocks are used to permit tissues to be cut thinly (4 to 5 μ m); cutting thin sections of tissue and staining them histochemically or immunohistochemically are necessary to permit tissues to be viewed adequately as to their structures (e.g., subcellular components and surrounding stroma) using a bright field microscope.

Over the last century, anatomists and pathologists have used fixation in 10% neutral buffered formalin (10% NBF) as the fixative of choice. Also, both human and veterinary pathologists have trained using fixation in 10% NBF so these professionals have been and are reluctant to change the microscopic appearance of diagnostic tissues by using a different type of fixation; in addition, the effects of tissue processing on the microscopic appearance of tissue has essentially been ignored in most studies. Because of the use of 10% NBF by pathologists, archives of paraffin blocks contain essentially paraffin blocks only fixed in 10% NBF. Thus, if retrospective studies use archival paraffin blocks to correlate the molecular features of diseases with the outcomes of diseases, the studies must be based upon using tissue fixed in 10% NBF.

Studies of how fixation in 10% NBF interacts with histochemical and immunohistochemical staining are very limited in number and most are based upon relatively long times of fixation in 10% NBF (≥ 36 hours). Current times of fixation in 10% NBF have been reduced to < 24 hours. Actually, little is known about fixation in 10% NBF and its interaction with tissue processing at any time of fixation, especially short times of fixation. Even less is known about how fixation of tissues in 10% NBF interact with more modern assays using immunohistochemistry, real time quantitative PCR, and techniques which depend upon the analysis of proteins extracted from paraffin blocks such as analysis by multiplex immunoassays or by mass spectrometry. In general, multiple antibody-antigen combinations are reported not to work in tissues fixed in 10% NBF, i.e., immunorecognition is almost lost completely for such antibody-antigen combinations as Ki67/MIB, ER α and PR, and partially lost for Bcl-2.

Several models have been developed to study the interactions of tissue fixation and immunorecognition, but most have viewed the problem in immunorecognition as being completely caused by fixation. Also, some of the models discussed in this special issue do not predict observations of the effects of fixation on frozen tissues fixed in 10% NBF, but not processed to paraffin blocks.

This article is a brief review of issues with using 10% NBF combined with tissue processing as a combined process to study biomarkers as identified by immunohistochemistry.

Keywords

formalin; fixation; immunorecognition; immunohistochemistry; models

Fixation, processing and staining are components of a process used to capture or “fix” in time relations among cells, and cellular (e.g., nucleoli) and extracellular (collagen in fibrosis) components of tissue. Therefore, by the first step of tissue processing, the scientist or pathologist tries to stabilize the apparent microanatomy of tissue (Arnold et al. 1996, Dapson et al. 2007, Eltoum et al. 2001, Fox et al. 1985) [Q5]. Although the visual products of fixation, which proceed to 4–5 µm paraffin sections, and immunohistochemical or histochemical staining are thought by some to represent closely the microanatomy of tissue, each combination of fixation, processing and staining are only compromises to permit visualization of the best static representation of a dynamic, living tissue (Grizzle 2001). The best compromise for fixation and tissue processing also should support various other methods of studying microanatomy and microphysiology, especially analysis by special histochemical stains, in situ hybridization, immunohistochemistry and microchemistry, e.g., mass spectrometry. Thus, the best compromise for fixation and processing should minimize changes in tissue following its removal from the living organism in both organizational structure and chemical composition (Grizzle 2001, Grizzle et al. 2007).

Staining of tissue sections produces a tissue product or picture in response to biological, chemical and/or physical inputs, or combinations of such inputs (Eltoum et al. 2001a,b, Grizzle et al. 2007). A physical input of fixation, e.g., heat, may occur as a frozen section is heated rapidly when the frozen tissue attaches to a warm (room temperature) microscope slide. Biological changes may occur and be observed when a ligand is added to living cells and there is a shift in the ligand’s receptor within cells (Fig. 1). Chemical changes usually are considered the major features of fixation whether they include removal of free water by 70–100% ethanol, addition of ethanol to form ethoxymethyl adducts, or addition of reactive chemical groups by 10% neutral buffered formalin (NBF) (Pearse 1968, Fox et al. 1985, Metz et al. 2006, Dapson 2007, Bogen et al. 2009, O’Leary et al. 2009, Otali et al. 2009). Each of these types of inputs, or combinations thereof, may be involved in illumination and clarification of the microanatomy of a wide range of tissues.

Over the past several decades, the disciplines of human and veterinary diagnostic pathology have been most dependent on fixation and therefore essentially have controlled the methods and study of “fixation.” In these two areas of study, the microscopic appearance of tissue microanatomy requires consistent results under the same conditions so that a reliable diagnostic separation can be made between one tissue type/cell type and others in paraffin sections of tissue fixed, processed and stained at widely different times (years), e.g., diagnostic separation of prostatic adenocarcinoma from benign prostatic hyperplasia or from prostatic intraepithelial neoplasia (Grizzle 2001). For successful diagnostic uses, each time a specific tissue is fixed, processed and stained under the same conditions, the microanatomy of tissue must appear the same regardless of whether or not the stained tissue represents an accurate representation of the microanatomy and/or contains artifacts of processing and staining.

One of the more important types of morphological diagnoses is the separation of benign from malignant tissues. The keys to this separation include the patterns of the organization of tissues as well as differences in nuclear features such as size and shape irregularities, nuclear to cytoplasmic ratios, irregular chromatin structures, and the size, number and color of nucleoli. All of these features are consistent in tissue fixed in 10% NBF and stained with hematoxylin and eosin (H & E) (Eltoum et al. 2001a,b, Grizzle 2001, Grizzle et al. 2007). [Q5.5] By contrast, the goal of the microanatomist is to view microanatomy so that staining of variously fixed and processed tissues represents as accurately as possible most aspects of the living tissue at a point in time (Grizzle 2001).

In addition to consistency of staining, the usefulness of 10% NBF as a general fixative is due in part to additional useful characteristics of 10% NBF. These include making insoluble many molecular species, preventing growth of microorganisms, maintaining tissue stability for decades of storage, preventing extensive swelling or shrinkage of tissue, and only slightly “hardening” (e.g., for easy cutting) of tissues after longer periods of fixation.

Training of pathologists has proceeded for many decades based on all of the features above and has made 10% NBF the fixative of choice for diagnostic pathology; it also has become the fixative about which the most is understood. During the last four decades, the increasing use of immunohistochemistry, in situ hybridization, and real time quantitative PCR in diagnostic pathology has led to the recognition that other methods of fixation, especially the use of ethanol as a fixative, may be superior for various modern diagnostic tests. Specifically, with the use of fixed tissues for determining diagnosis, prognosis and risk of disease, as well as for early detection, prediction leading to choice of therapies, and as surrogate end points for evaluating novel therapies, 10% NBF remains the primary fixative used in clinical medicine, even though its functions as a fixative are marginal for most of these more modern uses. This is especially true for immunohistochemistry in which 10% NBF has been shown to be a poor fixative (Fig. 2). Some problems with the use of 10% NBF as a fixative have been corrected by the introduction of other experimental techniques such as antigen retrieval (Shi et al. 2001) and the use of small amplicons in RT-Q-PCR (Steg et al. 2006,2007). Although some diagnostic pathologists have adopted ethanol based fixation for temporary periods, most pathologists continue to use 10% NBF as the fixative of choice. Thus, most archives of diagnostic paraffin blocks at medical and veterinary facilities contain primarily paraffin blocks in which the tissues were fixed initially in 10% NBF. Thus research with archival material must utilize methods that permit recovery of antigen recognition. Unfortunately, the optimal methods for antigen retrieval may vary with the antigen-antibody reaction to be evaluated.

Most efforts to understand fixation of tissues for paraffin embedment have involved long periods of fixation (e.g., > 48 h) and also have ignored the effects of the other steps in tissue processing on histochemical staining. Currently, fixation for diagnostic pathology typically is less than 24 h, and less is known and understood concerning such short fixation times except that H & E staining has been reported to be compromised with fixation in 10% NBF for 8 h or less (Dapson 2007).

The purpose of the scientific session of the Biological Stain Commission (BSC) in June 2007 was to present several of the newer models for studying the effects of fixation and the interaction of fixation with tissue processing. Of interest were the discussions on approaches to standardize immunohistochemistry. As discussed by Dr. Atha, the initial approaches of NIST in this area, focused on standardizing and comparing antibodies as an initial step in this process. Dr. Atha and colleagues found that commercially marketed antibodies to an antigen were quite variable as to their actual targets (Jakupciak et al. in press). Thus, antibodies should be evaluated carefully prior to their use. The lack of standardization of immunohistochemical techniques has proved to be a major impediment to wider use of immunohistochemistry in diagnosis as well as in research (Taylor 2006 [Q6]).

The 2007 Annual Scientific Session of the BSC focused on models of fixation and effects of fixation on immunohistochemistry as predicted by these models. The simplest model of fixation presented by Dr. Bogen evaluated single epitopes of diagnostic antigens, i.e., antibodies. Slightly more complex models presented by Dr. O’Leary evaluated the effects of fixation on simple proteins, RNA, and DNA, and the most complex model presented by Dr. Otali permits evaluation of the effects of fixation on intact cellular preparations. The complexity of the problem of the effects of fixation on immunorecognition is indicated by

the differences reported concerning the same antigen stained in DU145 cells versus SKOV-3 cells (Otalí et al. 2008). These results suggest that antigens may stain differently in two cell lines under the same conditions of fixation and tissue processing. This observation suggests that each type of cell may package antigens differently so that fixation and tissue processing may produce variable results among different cell lines when staining the same antigen with the same antibody. Thus, as is usually the case, the biology turns out to be much more complicated than initially was expected.

In the model presented by Dr. Bogen and colleagues (Sompuram et al. 2004, 2006a,b, Bogen et al. 2009), the epitope for Ki67/MIB-1 was stained with and without fixation in 10% NBF. [Q6.5] Dr. Bogen concluded that a product of the fixation bound to the Ki67/MIB-1 epitope and prevented immunohistochemical staining of the epitope. In his studies, other epitopes, such as the estrogen receptor, did not lose staining upon fixation by 10% NBF, but did lose all staining if a protein, i.e., casein, covered the epitope prior to fixation. Thus, the loss of staining of several antigens, e.g., ER and PR, was hypothesized to be due to cross-linking of their epitopes to casein. Application of this second protein, casein, was not required to abolish staining of Ki67/MIB-1. Thus, Dr. Bogen's model predicts that in frozen sections fixed in 10% NBF, there would be no staining with the Ki67-MIB-1 antibody. Figure 3 shows that there is relatively strong staining of 30% to 40% of nuclei in colorectal cancer after fixing a frozen section for 24 h in 10% NBF; similar staining also was noted in a case of breast cancer. Thus, we have conflicting results in the predictions in non-paraffin processed tissue based on the molecular model presented by Dr. Bogen.

Dr. O'Leary and colleagues (Mason and O'Leary 1991, Rait et al. 2004a,b, Fowler et al. 2008, O'Leary 2009) report a more complex model than that studied by Dr. Bogen's group. Dr. O'Leary's group suggests that there may be at least three ways by which formaldehyde can reduce immunorecognition of the epitopes of proteins. These include 1) "formation of extensive crosslinks by fixation reduces penetration of the primary antibody to the antigen target," 2) "reaction of formaldehyde with the target epitope reduces immunoreactivity," and 3) "the reaction with formaldehyde (of the antigen) causes changes in protein secondary structure and that reduces immunoreactivity." While these are the most likely, there probably are other possibilities. These include blocking the access of the secondary detection system to the primary antibody via structural changes upon fixation by aldehydes. Alternatively, a change in structure may inhibit the binding of the secondary detection system to the primary antibody. Also, fixation combined with specific steps in tissue processing may block immunorecognition. For example, once a protein has its associated free water removed and is surrounded by a hydrophobic environment, access to epitopes of primary antibodies and/or secondary detection systems may be severely compromised. To aid in these considerations, some of the more complex interactions of formaldehyde with the subgroups of proteins are shown in Fig. 4.

To study the issues of loss of immunorecognition upon fixation, Dr. O'Leary and colleagues (Mason and O'Leary 1991, Rait et al. 2004a,b, Fowler et al. 2008, O'Leary 2009) studied the effects of fixation on dilute solutions of ribonuclease A, a relatively small protein of about 14.4 KDa. After only 30 min incubation of a pure solution of ribonuclease A with 10% NBF, it was clear that fixation had caused the 14.4 KDa monomer to form dimers, trimers, tetramers and higher oligomers. This may indicate intermolecular bonding, i.e., cross-linking of molecules of ribonuclease A, and therefore changes in structure. The number of oligomeric forms of ribonuclease A was greatly reduced upon heating and the trimers and tetramers were reduced most, which is consistent with higher orders of oligomers being less stable to heat treatment. The issue of the relatively high lability of this bonding should be considered before it is considered covalent. Both of these results support the use of antigen retrieval for recovery of immunorecognition (Shi et al. 2001).

The O'Leary model gives results that are consistent with the observations reported by Otali et al. (2009). First, Otali et al. (2009) reported that fixation in 10% NBF without tissue processing resulted in a reduction in immunorecognition that progresses [Q7] with the time of fixation. Even fixation for 5 min in 10% NBF reduced immunorecognition and immunorecognition was recovered by antigen retrieval.

The Otali et al. (2009) model relies on use of intact cells in which antigens and their epitopes should be packaged similarly to their packaging in intact tissues. Consistent with this concept, it would not be surprising to obtain results that are different among various cell lines. In an intact tissue, the chemical changes produced by fixation may be constrained by the location of specific molecules within a cell and/or by the biology of the cell. Consistent with this concept, this model detected differences in the effects of fixation by 10% NBF on different cell lines in which antigens may be packaged and located differently.

The results presented by Otali (2009) are partially consistent with another observation using the ribonuclease model (Fowler et al. 2008), i.e., that dehydration with ethanol inhibits immunorecognition. Because the primary sample after antigen recovery following ethanol remained in the form of monomers, however, this result would not imply that alcohol treatment after fixation reduced immunorecognition.

According to the Otali (2009) model, fixation without tissue processing reduces immunorecognition, but does not abolish it. It is only when fixation of DU145 cells is combined with establishment of a hydrophobic environment that immunorecognition of Ki67 is essentially lost. It should be noted that establishing the hydrophobic environment alone, even without fixation, is just as effective in abolishing immunorecognition of Ki67. In a second model of intact tissue using frozen sections, similar results regarding establishing a hydrophobic environment were obtained for Ki67 and Bcl-2 upon combining fixation with tissue processing (Stockard et al. unpublished).

Any model that is developed to study or characterize the effects of fixation and/or tissue processing on immunorecognition should be able to explain how Ki67/MIB-1 can be detected by immunostaining in frozen sections of tissue fixed for 24 h in 10% NBF before staining, but not in paraffin sections from tissue fixed similarly for 24 h in 10% NBF. In addition, the cellular variability noted may indicate that fixation, tissue processing and immunostaining may have to be optimized for each tissue and antibody-antigen combination (Wang et al. 2008).

Biographies

William E. Grizzle, M.D., Ph.D., received a BA degree in chemistry and physics from Harvard University. He completed coursework for a Master's Degree in physics at Georgetown University while working for the Atomic Energy Commission/Navy with Naval Reactors. He subsequently received a Ph.D. followed by an M.D. from Johns Hopkins University. He is board certified in anatomic and clinical pathology with residencies in pathology at Vanderbilt University and the University of Alabama at Birmingham (UAB). He has been a member of the faculty of UAB since 1981 and currently is Professor of Pathology and Head of the Program in Translational Research in Neoplasia. His research interests primarily are in the use of biomarkers in clinical medicine and research on factors such as tissue processing and fixation that affect the use of biomarkers in early detection, determining prognosis, predicting effectiveness of therapies in risk assessment and as surrogate end points for evaluating novel therapies such as gene therapy.

Donald Atha received his undergraduate degree in chemistry in 1970 from the University of California in San Diego and his graduate degree in biochemistry in 1974 from the University of Virginia in Charlottesville. He worked as a postdoctoral fellow and instructor from 1974 to 1978 in the Department of Zoology of the University of Texas in Austin and as a staff fellow from 1978 to 1981 at the American Red Cross Blood Services in Bethesda, concentrating on the structure and function of plasma proteins. From 1981 to 1987, Dr. Atha continued work on heparin and anti-thrombin as a research associate in the Whitaker College of MIT and as an instructor in the Department of Pathology of Beth Israel Hospital. Since 1987, he has served as a research chemist at the National Institute of Standards and Technology (NIST) in Gaithersburg, MD, concentrating on chromatographic, calorimetric and spectral techniques for separation and chemical characterization of biologically important macromolecules, with emphasis on heparin and DNA. His recent work has included development of improved methods for the analysis of p53 single point mutations, fragile X syndrome and telomerase using capillary electrophoresis.

Steven Bogen M.D., Ph.D., is the Medical Director of the Clinical Chemistry Laboratory at the Boston University Medical Center. He received his M.D. from the University of Chicago Pritzker School of Medicine, completed his Ph.D. in immunology at the Weizmann Institute of Science, and residency training in anatomic and clinical pathology at the Brigham and Women's Hospital, Boston, MA. Dr. Bogen's research centers on the development of new technologies for clinical diagnostics. He is the inventor and architect of the Artisan® instrument for special stains, which is sold by Dako Corporation. He also is the co-inventor of a new technology for mass produced, standardized immunohistochemistry controls using peptides in lieu of tissue sections or cell lines; this system has been commercialized by ThermoFisher Corporation. His current research is in the areas of gammopathies, such as amyloidosis AL and multiple myeloma, and the development of new cancer diagnostics.

Timothy J. O'Leary received his Ph.D. in physical chemistry from Stanford University and his M.D. from the University of Michigan prior to completing a residency in pathology and a fellowship in chemical physics at the National Institutes of Health. In 1986, he moved to the Office of Biologics in the FDA Center for Drugs and Biologics, where he remained for one year before assuming the chairmanship of the Department of Cellular Pathology and Genetics at the Armed Forces Institute of Pathology. During 18 years of service at the AFIP, Dr. O'Leary has made important contributions to pathology research and practice, particularly within the emerging discipline of molecular genetic pathology, a subspecialty in which he was certified in 2002. In late 2003, after being recalled to duty with the Public Health service, he served briefly as Director of the Division of Immunology and Hematology Devices of the FDA. In March 2004, he became Director of the Biomedical Laboratory Research and Development Service of the Department of Veterans Affairs, and in June 2005 assumed additional duties as Director, Clinical Science Research and Development Service. In this role, he directs the VA Cooperative Studies Program, the nation's first nationwide multi-site clinical trials network. Since March 2008, he has served as the Acting Deputy Chief Research and Development Officer for the VA. Dr. O'Leary has served on a number of advisory groups including the Molecular Genetic Pathology Test Committee of the American Board of Pathology, the American Board of Medical Genetics, the HHS Clinical Laboratory Improvement Advisory Committee, and the FDA Pathology and Hematology Devices Panel. He has edited three books and authored or co-authored over 150 journal articles and book chapters.

Dennis Otali, B.S., M.S., is a resident of Uganda, where he received his undergraduate degree at Makerere University. He subsequently taught histology at Faculty of Medicine Makerere University. He currently is a Ph.D. student in the Department of Biology, School of Natural Sciences and Mathematics, UAB, studying insecticide resistance in mosquitoes.

This manuscript is part of his dissertation for his Master's Degree in Biology at UAB. His research was completed in Dr. Grizzle's laboratory.

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Fig. 1.

Changes in the biology of a specimen can modify the immunohistochemical appearance of tissue. Panel A1) Membrane expression of DU145 cells stained with an antibody to EGF receptor (straight black arrow). Panel A2) Change in staining for EGFr after exposure of DU145 cells to the ligand of the EGF receptor, EGF, for 2 h. Striped arrows indicate a granular expression of EGFr in the perinuclear area after exposure to EGF; there is little membrane expression remaining. Panel B1) Staining of unstimulated DU145 cells stained for pERK1/2 (double-headed black arrow); a downstream signal molecule of the activated EGF receptor. Panel B2) Increased phenotypic expression of pERK1/2 (thin black arrows with circular knob) in these cells following continuous exposure to EGF for 2 hr and subsequent activation of the EGF receptor. Curved arrows point to mitotic cells. Panels A1, A2, and B2 x400, Panel B1 x200.[Q9]

Fig. 2.

Typical staining of each antigen following immunostaining after fixation of aliquots of the same tissue in each of seven fixatives. Note the similar patterns for keratins (cytoplasmic antigens) and p53 (a nuclear antigen) by contrast to p185^{erbB-2} and TGF α . [Q10] Bars represent standard error of the mean (SEM).

Fig. 3. Ki67/MIB-1 staining of an area of colon cancer in a frozen section fixed [Q11] 24 h in 10% NBF. Note definite staining of the nuclei of the colorectal cancer with Ki67/MIB-1 (black arrows) independent of 24 h fixation in 10% NBF; there is no staining of the surrounding stroma. Similar staining with Ki67/MIB-1 was noted in an area of a frozen section of breast cancer fixed [Q12] for 24 h in 10% NBF (results not shown).

Fig. 4.
Some of the more common reactions of formaldehyde or ethanol with protein side chains (modified for this article from Dapson 2007).