

Applying immunohistochemistry to alcohol-fixed paraffin-embedded tissues: an innovative technique to reduce use of formaldehyde

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Summary. Recent advances in molecular biology and pathology have opened new opportunities for refining our knowledge of pathophysiologic events and biomarkers. Particular interest in applying these novel methods to current and archived tissues collected in experimental and epidemiological/clinical studies is evident. Until now, it has not always been possible to use archived alcohol-fixed paraffin-embedded (AFPE) tissues for immunohistochemistry (IHC), because AFPE slices and blocks were not often amenable to standard IHC methods. In order to solve this problem, we developed a simple method of post-fixation, which allows to use, on AFPE slices, standard IHC protocols already used for formalin-fixed paraffin-embedded (FFPE) samples. For the assessment of post-fixation processing and to test the feasibility of IHC, we selected the spleen from Sprague-Dawley rats as a demonstrative tissue. Antibodies to PAX5, CD3, CD68 and Ki-67, were tested on FFPE, AFPE and AFPE post-fixed spleen samples. The specificity of antibodies to bind different epitopes expressed in spleen tissue was maintained in FFPE and AFPE post-fixed sections, according to anatomical localization. Post-fixation of AFPE samples did not affect tissue morphology and IHC results were comparable to the FFPE sections in terms of sensitivity, specificity and intensity of staining. In addition to providing an opportunity to use archived tissues, this new post-fixation method would dramatically reduce the use of formaldehyde during histopathology procedures, thus minimizing worker exposure to this dangerous carcinogenic substance.

Key words: alcohol-fixation, IHC, AFPE post-fixation, FFPE, formalin fixation

«APPLICAZIONI D'IMMUNOISTOCHEMICA SU TESSUTI FISSATI IN ALCOL E INCLUSI IN PARAFFINA: UNA TECNICA INNOVATIVA PER RIDURRE L'USO DI FORMALDEIDE»

Riassunto. I recenti progressi nel campo della biologia molecolare e della patologia hanno aperto nuove opportunità per affinare le nostre conoscenze sugli eventi patofisiologici e sui biomarker. Appare evidente il particolare interesse nell'applicare queste nuove tecniche sui nuovi e sui vecchi tessuti archiviati durante studi clinici, epidemiologici e sperimentali. Finora, non è sempre stato possibile utilizzare tessuti fissati in alcol e inclusi in paraffina (AFPE) per l'immunoistochimica (IHC), perché blocchetti e sezioni AFPE non risultavano spesso ottimali per l'utilizzo di metodi standard di IHC. Per risolvere questo problema, abbiamo sviluppato un semplice metodo di post-fissazione, che permette di utilizzare, su sezioni AFPE, protocolli standard di IHC già in uso per campioni fissati in formalina e inclusi in paraffina (FFPE). Per valutare il processo di post-fissazione e testarne l'affidabilità per l'IHC, abbiamo scelto come tessuto di prova la milza di ratto Sprague-Dawley. Sono stati testati anticorpi contro PAX5, CD3, CD68 e Ki-67 su campioni FFPE, AFPE e AFPE post-fissati. La specificità degli anticorpi nel legare i differenti epitopi espressi nel tessuto

della milza è stata mantenuta sia nelle sezioni FFPE che in quelle AFPE post-fissate, in accordo con la loro localizzazione anatomica. Il processo di post-fissazione dei campioni AFPE non ha intaccato la morfologia del tessuto e i risultati dell'IHC sono stati comparabili a quelli di campioni FFPE in termini di sensibilità, specificità e intensità della colorazione. Oltre a fornire l'opportunità di utilizzare i tessuti archiviati, questo nuovo metodo di post-fissazione riduce enormemente l'uso di formaldeide durante le procedure istopatologiche, minimizzando l'esposizione dell'operatore a questa pericolosa sostanza cancerogena.

Parole chiave: fissazione in alcol, IHC, post-fissazione AFPE, FFPE, fissazione in formalina

Abbreviations: CMCRC, Cesare Maltoni Cancer Research Center; IHC, Immunohistochemistry; FFPE, formalin-fixed paraffin-embedded; AFPE, alcohol-fixed paraffin-embedded; HE, Hematoxylin Eosin; NBF, neutral buffered formalin; HIER, Heat Induced Epitope Retrieval; SD, Sprague-Dawley; PALS, PeriArteriolar Lymphoid Sheaths; Hope, Hepes-glutamic acid buffer mediated Organic solvent Protection Effect.

Introduction

Toxicological studies are of great value for providing preclinical information as well as data, important to developing strategies to reduce or prevent human exposures to agents that increase risks of diseases and toxicologic effects such as cancer. Pathological analyses have been a key element in these observational studies, but until now, it has not been possible to use the large numbers of alcohol-fixed paraffin-embedded (AFPE) tissues, archived from past studies, for increasing our knowledge through application of immunohistochemistry (IHC) (1). This is because AFPE tissue slices and blocks were not readily amenable to current IHC methods. Consequently, the vast information value of these resources from studies of experimental interventions are not available for review and utilization in clinical and other evaluations through IHC.

IHC also provides improved tools for objective analyses in pathology, amplifying expert judgement of morphological review. This reduces the potential for differences in interpretation of experimental results by providing molecular information in addition to

standard diagnostic methods used in many protocols (1).

Histopathological assessment plays a central role in toxicological pathology, and eventual utilization in human health hazard identification. Conventional morphological analysis for evaluating the nature and biological behaviour of specific lesions primarily relies on the experience and judgment of the pathologist rather than on purely objective criteria (2-3). Moreover, the ability to extract further information does not displace the importance of expert judgement and experience but rather amplifies the information available to all involved in such assessments. Morphology is important in terms of evaluating relevance to human diseases of characteristic or even pathognomonic features, but it is now recognized in clinical medicine that additional molecular information can add important data for defining the nature of observed histopathology and likely pathophysiology. Finally, application of IHC and other molecular methods allows experimental toxicology to take advantage of tools that are increasingly in common use in the clinical medicine. For example, IHC is essential in human pathology for characterizing hemolymphoreticular neoplasms. This then enhances the ability to draw evidence-based inferences from experimental to human situations, such as chemical exposures (3).

To date, our ability to use IHC and other new tools is often limited by the choice of methods for tissue fixation. Clearly there is no "one fixative fits all" situation for immunohistochemistry (4-5). The goal of fixation is to achieve rapid stoppage of vital cellular

processes to prevent or suspend post mortem degradation (e.g., autolysis) while maintaining minimal alterations of cellular and intracellular shape, volume, spatial and molecular relations. Fixation processes may be physical or chemical. A common example of physical process is the rapid freezing of samples, which is suitable for analyses of RNA, DNA, lipids and mucopolysaccharides. However, freezing does not provide optimal preservation of tissue morphology. Chemical processes include cross-linking fixatives, such as formaldehyde and glutaraldehyde; denaturing fixatives, such as ethanol and methanol; oxidising agents especially used for electron microscopy; and other fixatives such as mercurials, picrates, and HOPE (Hepes-glutamic acid buffer mediated Organic solvent Protection Effect) Fixative (6-8).

Formaldehyde [HCHO] is the most commonly recommended and used fixative for routine histology and IHC, usually as a 10% [\sim 4% HCHO] neutral buffered form (NBF), because of low cost, ready availability and preserves tissue morphology (9). Its basic mechanism of fixation consists in forming addition products between the formaldehyde and the uncharged reactive amino groups ($-NH$ or $-NH_2$) inducing protein-DNA and protein-protein cross-links. For IHC analysis formalin-fixed paraffin-embedded (FFPE) specimens require antigen retrieval techniques, by Heat Induced Epitope Retrieval (HIER) or strong alkaline hydrolysis, to remove this cross-linking blockage, allowing targeted epitopes to be exposed and bound by specific antibodies (4, 10). Despite NBF being the most common fixative used for IHC, it also presents limits for molecular analyses because it quickly degrades and modifies the chemical structure of nucleic acids and proteins (11-13). Furthermore, formaldehyde represents an important health issue of professional users because of its toxicity and carcinogenicity, and should be replaced (14-18).

Buesa (2008) (19) and Benerini Gatta *et al* (2012) (8) pose "The search for an alternative to formalin fixation, which offers better technical performance and greater protection for health workers, is unavoidably needed". Likewise Zanini *et al* (2012) (6) stress "the carcinogenicity of formaldehyde for humans renders its substitution in Pathology Departments necessary".

Alcohol-based fixatives are considered as valid alternatives to formalin, especially for tissue preservation and storage and molecular analyses (6, 19-20). Alcohol-fixation is based on interaction of alcohol with molecular hydrophobic areas: alcohol removes and replaces water in tissues, causing changes in tertiary structures of proteins (4, 21). Alcohol-fixation, as demonstrated by many researchers, maintains the morphology of tissues (22), and preserves macromolecules in paraffin embedded tissues (20, 23). However, at present, epitope retrieval methods for IHC analysis in AFPE tissues have not yet been developed and validated (4).

In our laboratory, for example, it is especially important to characterize proliferative lesions of hemolymphoreticular tissues from neoplastic ones, particularly to better evaluate potential carcinogenic risks of chemicals to humans. Out of 209 carcinogenesis bioassays at our laboratories, 49 (23%) were carcinogenic (24) and among them 8 increased hemolymphoreticular malignancies. Out of these, 3 were caused these lesions in males and females-formaldehyde (16), mancozeb (25), and di-isopropyl-ether (26) and 5 only in females-toluene (27), methyl alcohol (28), methyl tert-butyl ether (29), tert-amyl-methyl-ether (26), and aspartame (30-32). A Pathology Working Group convened by the U.S. National Toxicology Program (NTP), National Institute of Environmental Health Science (NIEHS) to independently evaluate these lesions, and decided some diagnostic differences for certain lymphomas localized in lungs of experimental animals (33).

Due to these diagnostic differences of opinion, possible economical impact, and significantly likely health risks of worker and consumer exposure to these high volume chemicals (MTBE, methanol, ETBE), we considered it as necessary to further characterize these lesions using IHC techniques. Problematically though alcohol fixation for these tissues is not suitable for IHC. In this paper we describe a new post-fixation method that allows the use of IHC assays on AFPE samples. For development and validation of this method, we used spleens from Sprague-Dawley (SD) rats as demonstrative tissue, since in spleen all different hematopoietic cellular lineages are represented. The overarching goal was to determine if this method

would permit IHC characterization of different experimental tissues archived in our laboratory.

Materials and methods

Animals

For this study, tissues were taken from ten ~30-week old Sprague-Dawley rats from the colony used at the Cesare Maltoni Cancer Research Center (CMCRC) over the past 40 years (32). Animals were cared for according to Italian law (34), following European recommendations, regulating the use and the human treatment of animals for scientific research.

Tissue examined

For the assessment of methodologies for post-fixation processing and IHC, we selected the spleen as a demonstrative tissue because of the heterogeneity of cell types, including hemolymphopoietic cell lineages, such as B- and T-lymphocytes, their precursor, macrophages, and myeloid cells. Each of these cell populations has a specific anatomical distribution pattern in spleen. IHC assays, using specific antibodies (35), give distinct and replicable patterns of staining for these cellular populations (1, 36-38).

Collection of samples

During necropsy half of each spleen was fixed in 70% alcohol (Solvanol, VITAL srl), and the other half fixed in 10% NBF (Biochimica srl, Italy) and maintained at room temperature for 24-48 hours before transfer and storage in 70% alcohol. The two halves were then processed and embedded in paraffin blocks following CMCRC procedures (39). Sections were cut at 1-3 μ m and collected in polylysine coated slides and air dried: we obtained eleven sections from AFPE tissues and 6 sections from FFPE tissues. One section, from FFPE and AFPE blocks, was stained with HE and evaluated through optical microscopy for pathology.

Fifteen sections from each spleen, to be evaluated with IHC, were divided as: 1) FFPE slices; 2) stan-

Table 1. Post-fixation protocol for slices of alcohol-fixed paraffin-embedded (AFPE) tissues

Solvent	Time/ n. of step
1. Xylene	10 min/ 3 times
2. 100% Alcohol	5 min/ 2 times
3. 95% Alcohol	5 min/ 2 times
4. 80% Alcohol	5 min/ 2 times
5. 70% Alcohol	5 min/ 2 times
6. PBS 1X	5 min/ 2 times
7. Distiller water	5 min/ 2 times
8. 10% NBF	30 min 4°C
9. 70% Alcohol	18-24 h

dard AFPE slices; and 3) AFPE slices treated with a post-fixation in 10% NBF, following the procedure described in Table 1.

Pre-immunohistochemical treatment

FFPE and AFPE post-fixed sections were submitted to antigen retrieval at 65°C in Dako PTLINK (DakoCytomation, Glostrup, Denmark). Slides were placed in EnVision Flex Target Retrieval Solution High pH (DakoCytomation, Glostrup, Denmark) at 92°C for 5 min. When temperature cooled to 65°C slides were set into jar/tank containing room temperature EnVision Flex Wash Buffer (DakoCytomation, Glostrup, Denmark) for 15 min. Conversely, AFPE tissues were only deparaffinized and rehydrated by passing samples through alcoholic solutions, from 95% to 70% and distilled water, before IHC staining.

Antibodies selection

We selected a panel of rat antibodies reacting with epitopes expressed in the main cell populations of the spleen according to the literature data (1, 36, 38). PAX 5 is a nuclear antigen expressed by pro-, pre- and mature B cells, and stains follicles and marginal zone of spleen. CD3 is a membrane antigen expressed by T cells, and stains the PeriArteriolar Lymphoid Sheaths (PALS) of white pulp. CD68 is a cytoplasmic antigen expressed by monocytes/macrophages

Table 2. Antibodies used for IHC analysis in Sprague-Dawley rat spleen

Antibody (Dilution)	Cell Marker	Cellular localization	Source	Product number	Clone
PAX 5 (1:50)	Pro, pre, and mature B	Nuclear	Dako Cytomation	IR650	DAK-Pax5
CD3 (1:150)	Early T Cell	Membrane	Thermo SCIENTIFIC	RM-9107	SP 7
CD68 (1:100)	Myeloid Cell	Membrane	AbD serotec	MCA341R	ED 1
Ki-67 (1:200)	Growth fraction of normal and neoplastic cells	Nuclear	Thermo SCIENTIFIC	RM-9106S	SP 6

and dendritic cells, staining red pulp and marginal zone. Ki-67 is a nuclear antigen expressed by dividing cells, staining normal and abnormal proliferating cells of spleen tissue (Table 2).

Immunohistochemical analysis

To all slices (FFPE, AFPE and AFPE post-fixed), 200 µl 10% Fetal Calf Serum was added; samples were incubated in a humidified chamber for 15 minutes to block non-specific binding sites. Endogenous peroxidases were quenched for 15 minutes with 200 µl Peroxidase-Blocking Reagent (DakoCytomation, Glostrup, Denmark), then incubated for 1 hour with primary antibody at dilutions reported in Table 2. All antibodies were diluted with Dako Real Antibody Diluent (DakoCytomation, Glostrup, Denmark). According to manufacturer's instructions, we applied 200 µl EnVision Flex+ Mouse Kit (DakoCytomation, Glostrup, Denmark) to the slices for 30 minutes. Slices were rinsed in Wash Buffer (TBS-Tween 20%) after each step above described. Afterwards, slices were incubated in 200 µl diaminobenzidine hydrochloride DAB (DakoCytomation, Glostrup, Denmark) for 5 minutes. Finally, slices were rinsed in distilled water. Sections were counterstained with EnVision Flex Hematoxylin (DakoCytomation, Glostrup, Denmark), and washed in distilled flowing water for 2 minutes. Cover slips and mountant were applied for optical microscopy analysis.

Results

Hematoxylin Eosin (HE) evaluation

HE sections were microscopically examined and the spleen appeared normal; morphological distinct compartments, white and red pulp, were distinguishable (Figure 1). No evident differences in morphology or quality of staining with HE between FFPE and AFPE tissues were observed.

Immunohistochemical evaluation

Antibodies to PAX5, CD3, CD68, and Ki-67 were tested in every slice for all three types of samples: FFPE, AFPE, and AFPE post-fixed tissues. Optical microscopy evaluation was performed on each sample and photomicrographs was taken.

As expected, in FFPE samples antibody binding was specific for different cell populations identified by antibodies given in Table 2. On the contrary, AFPE sections, with no further treatment, were not responsive to IHC staining using any of the selected antibodies; staining was weak or not present at all.

In the AFPE post fixed slices, IHC staining results were comparable to the FFPE sections in terms of sensitivity, specificity and intensity of staining. In fact the same cell populations were precisely identifiable through the antibodies of our panel (Table 2) in both FFPE and AFPE post-fixed slices (Figure 2).

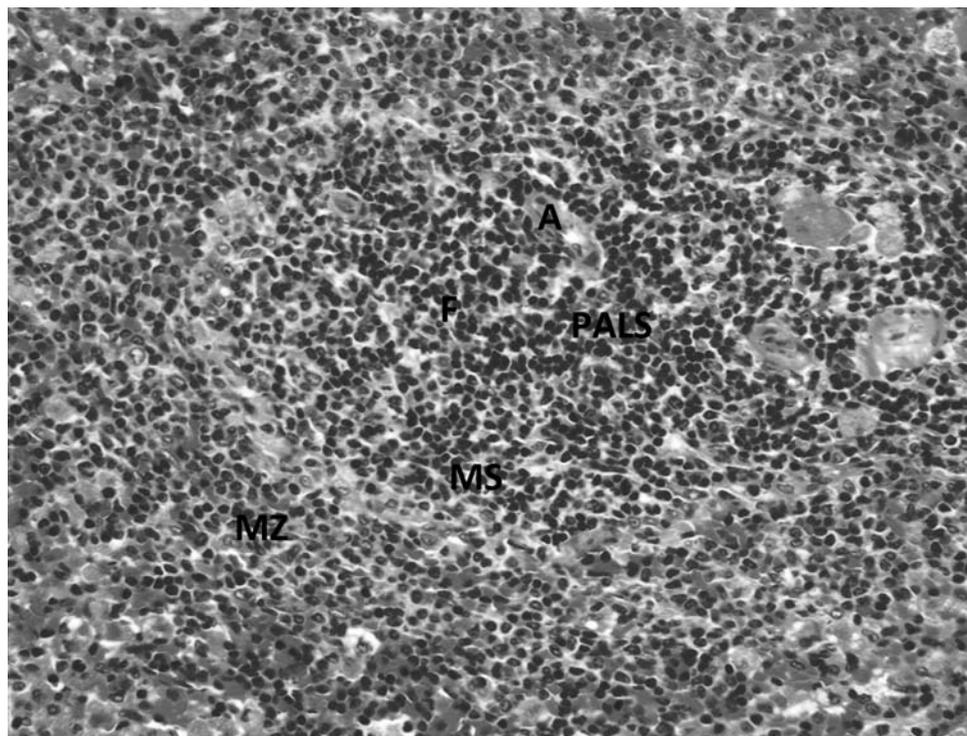


Figure 1. Sprague-Dawley rat, 30 weeks old. Normal spleen, formalin-fixed and paraffin-embedded, stained with Hematoxylin Eosin. (400X). The white pulp is divided in follicle (F), Periaarteriolar Lymphoid Sheaths (PALS) around the central arteriole (A), marginal sinus (MS) and marginal zone (MZ).

Discussion

Direct IHC analysis in AFPE tissues is not applicable following standard IHC procedures, probably because of alcohol interaction with protein hydrophobic moieties modifying the tertiary structure of the protein, likely preventing correct exposures of antigen epitopes (4). Our results on AFPE sections confirm a low quality of staining when routine IHC procedures were applied to these slides, consistent with the literature. To overcome this problem, we developed a new method of post-fixation, which allows using on AFPE slices the same standard IHC protocols already in use for FFPE samples. As shown in results, this method doesn't adversely affect tissue morphology, and preserves immunophenotype when compared to FFPE sections. Moreover, specificity of antibodies to bind different epitopes expressed in spleen tissue is maintained in both FFPE and AFPE post-fixed sections, according to their anatomical localization.

Furthermore, our findings show the advantages of alcohol-fixation over FFPE, that in the first processing stage, it preserves the secondary structure of proteins and, after post-fixation, allows identifica-

tion of specific molecules by IHC. In addition, the use of alcohol fixation results in improved preservation of DNA and RNA, increases the ability to carry out additional molecular analyses that enable application of other technologies such as laser cytometry and in situ PCR (40).

The use of these tools on historical archives of stored tissues and block from major toxicological studies is of considerable importance, and opens additional opportunities to retrieve valuable information from a retrospective analyses of these samples. In studies of chemical carcinogenesis, additional IHC markers for proliferation, apoptosis and specific tumor proteins may be used to distinguish hyperplasia from neoplasia, and determine specific tumor origin/type or progression of a given neoplasm (1, 38). This would be highly relevant for confirming diagnosis based solely on HE morphological analysis. For example, in studies of chemically induced carcinogenesis, the use of IHC may confirm or reject the clonal origin of neoplasms (3, 41-45). This innovative post-fixation technique potentially allows us to perform IHC analysis with standard protocols in our AFPE samples, represented by an archive of millions of

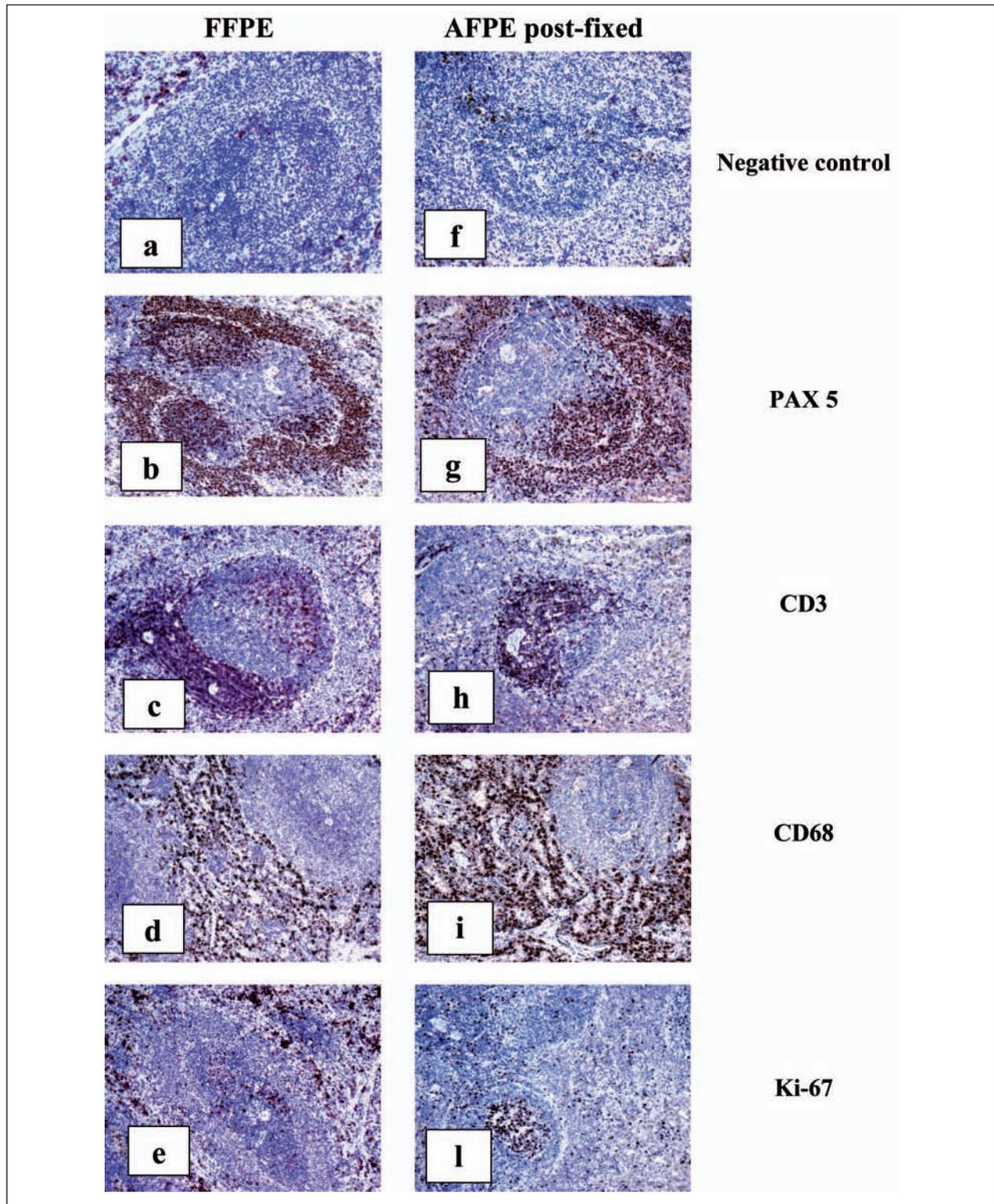


Figure 2. Normal spleen of Sprague-Dawley rat, 30-weeks old. IHC comparison between formalin-fixed paraffin-embedded (FFPE) and alcohol-fixed paraffin-embedded (AFPE) post fixed samples (200X). Negative control (without primary antibody), PAX 5, CD3, CD 68, and Ki-67.

treated or untreated SD rat tissues, preserved in paraffin blocks (16).

Analysis on archived 10–20 years old samples are underway and in the next months we will have the results. Furthermore, the assessment of a method for old samples will allow us to reduce the need to redo past and archived experimental bioassays.

Finally we observe the importance of the reduction in hazards to toxicologists and pathologists in terms of avoiding exposures to the known human carcinogen formaldehyde. Significantly, this new postfixation method could dramatically eliminate or considerably reduce the use of formaldehyde during histopathology procedures and minimize worker exposures to this dangerous chemical, which is an important health risk often underestimated by pathologists and technicians (6, 8).

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