Evaluation of the Value of Frozen Tissue Section Used as "Gold Standard" for Immunohistochemistry

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Abstract

To examine the use of acetone- or ethanol-fixed frozen tissue sections as the "gold standard" for immunohistochemical analysis, we evaluated frozen sections with various conditions of fixation and antigen retrieval (AR). Fresh human tissues were frozen in OCT. An adjacent tissue block was fixed in 10% neutral buffered formalin (NBF) and paraffin embedded (FFPE). Frozen sections were fixed by 6 protocols: acetone, ethanol, NBF (2 durations), and NBF + calcium chloride (2 durations). AR was used for all NBF-fixed sections.

More than half of the antibodies (16/26 [62%]) showed immunohistochemical results indistinguishable between acetone- and NBF-fixed sections; 8 (31%) showed better immunohistochemical signals following NBF and AR; 2 gave better immunohistochemical results for acetone-fixed sections. Most cytoplasmic proteins (10/13) showed comparable immunohistochemical signals between acetone- and NBF-fixed sections. For nuclear proteins, NBF-fixed sections gave better immunohistochemical signals than did acetone-fixed sections. In most cases, NBF yielded stronger signals with less background and better morphology. The data do not support the use of acetone-fixed frozen tissue sections as the gold standard for immunohistochemical analysis. In evaluating new antibodies, a combination of acetone- and NBF-fixed frozen sections should be used, although in practice, FFPE tissue sections may serve as the standard for most antigens for immunohistochemical analysis.

Fresh cell smears or frozen tissue sections have been the standard for immunofluorescence and immunohistochemical analysis from the inception of these methods in the mid 20th century. Subsequently, with the advent of immunoperoxidase-labeled antibodies, there was a growing focus on the application of immunohistochemical analysis to archival formalin-fixed, paraffin-embedded (FFPE) tissue sections because in practice, FFPE blocks represent the material available in the diagnostic setting and also because archival collections of FFPE tissue blocks form invaluable resources for translational studies of cancer and other diseases. Despite the fact immunohistochemical methods with antigen retrieval (AR) are now applied to FFPE tissues for almost all diagnostic work and for many research studies,[1-3] fresh cell and tissue samples are still regarded by many as the "gold standard" for validating immunohistochemical results. This assumption especially applies when evaluating new markers and new reagents to establish the "true" immunohistochemical findings. The unspoken rationale for this practice is that "formalin fixation is bad"; therefore, absence of formalin fixation must be good; but the argument is flawed.

Arising from the widespread use of FFPE and AR are isolated reports of discrepancies of immunohistochemical results between frozen and FFPE tissue sections. For example, Yamashita and Okada[4] examined the immunostaining results of 22 antibodies comparing acetone- and aldehyde-fixed frozen tissue sections and found that most antibodies showed stronger intensity of immunohistochemical staining for aldehyde-fixed frozen tissue sections after the AR treatment, compared with findings obtained in acetone-fixed tissue sections. In particular, a total of 11 antibodies (50%) that gave negative immunohistochemical staining results using acetone-fixed

frozen tissue sections yielded positive staining with aldehyde-fixed frozen tissue sections with AR.

In the course of other studies, we also have observed weak or absent staining for some antibodies tested on acetone-fixed fresh cell or tissue sections. For example, a newly developed polyclonal antibody to glucose-regulated protein (GRP) 78 showed no detectable reaction in an acetone-fixed fresh cell line specimen but showed positive staining in formalin-fixed preparations of the same fresh cell sample after AR treatment. These observations led us to question the long-held belief in the reliability of acetone- or alcohol-fixed fresh tissue sections when used as the "gold standard" for immunohistochemical staining.

The present study was designed to evaluate the concept of the gold standard by comparing immunohistochemical staining results of fresh human tissue sections, Cytospin preparations, cultured cell pellet FFPE blocks, and FFPE tissue blocks, each fixed by a panel of different protocols, and using AR pretreatment when formalin was used for fixation. A Western blotting technique was used to confirm the presence of selected proteins in comparison with immunohistochemical localization in the corresponding cells and tissues.

Materials and Methods

Human Tissue Samples

Fresh human tissue samples of breast, colon, adrenal gland, and bladder cancers; melanoma; and lymph node **Table 1** were obtained from surgical procedures at the Norris Cancer Hospital and Research Institute, University of Southern California Keck School of Medicine (USC), Los Angeles, and promptly divided into 2 parts: one part was immediately snap frozen, embedded in OCT compound (Miles Laboratories, Elkhart, IN), and stored at –70°C; a second part was fixed in 10% neutral buffered formalin (NBF) overnight at room temperature, following which routine paraffin embedding was carried out using an automated processor (Tissue Tek VIP, Sakura Finetek, Torrance, CA). Frozen and FFPE tissue sections (5 μm) were cut with a cryostat or a microtome, respectively, and mounted on commercially available charged slides (Fisher Scientific, Pittsburgh, PA). This study of anonymous human archival tissue specimens was exempted under 45 CFR § 46.101 (b) and was approved by the USC Institutional Review Board (IRB No. 009071).

Table 1
Primary Antibodies, Tissue Samples, and Antigen-Retrieval Immunohistochemical Methods

Antibody/Type (Clone)	Cell/Tissue*	Source†	Concentration	Antigen-Retrieval Protocol	Detection System
ER/M (6F11, Ab-12)	Normal breast (S)	Lab Vision	1:100	CA	ABC/DAB
Ki-67/M (MIB-1)	LN (S)	DakoCytomation	1:500	CA	ABC/DAB
p53/M (Ab-2)	Colon ca (F)	BioGenex	1:5,000	CA	2-step
p27/M (Ab-1)	LNCaP (F)	Lab Vision	1:400	Citrate buffer, pH 6	2-step
Rb protein/M (G3-245)	Bladder ca (F)	BD Biosciences	1:200	CA	ABC/DAB
p21/M (Ab-1)	MCF-7; bladder ca (F)	EMD	1:100	Citrate buffer, pH 6	2-step
Pan-keratin/M (AE1, AE3)	LN (S)	Ventana	Prediluted	CA	ABC/DAB
S-100/M (4C4.9)	Melanoma (S)	Biocare	1:200	CA	ABC/DAB
Vimentin/M (V9)	Melanoma (S)	Chemicon	1:15,000	CA	ABC/DAB
CK7/M (OV.TL12/30)	LN (S)	DakoCytomation	1:50	CA	ABC/DAB
CK20/M (KS 20.8)	Colon ca (F)	DakoCytomation	1:50	CA	ABC/DAB
Desmin/M (D33)	Colon ca (F)	DakoCytomation	1:100	CA	ABC/DAB
Actin/M (IA4)	Colon ca (F)	DakoCytomation	1:600	CA	ABC/DAB
Factor VIII antigen/P (F8/8b)	Colon ca (F)	DakoCytomation	1:600	CA	ABC/DAB
CEA/M (Col-1)	Colon ca (F)	Invitrogen	1:30	CA	ABC/DAB
GRP 78/P (H-129)	C42B and breast ca (S)	Santa Cruz	1:200	CA	ABC/DAB
Melanosome Melan A/M (MC-7C10)	Melanoma (S)	Cell Marque	1:80	CA	ABC/DAB
Survivin/P	C42B (F)	NeoMarkers	1:300	CA	ABC/DAB
bcl-2 Oncoprotein/M (124)	LN (S)	DakoCytomation	1:20	CA	ABC/DAB
CD45/M (2B11/PD7/26)	LN (S)	DakoCytomation	1:20	CA	ABC/DAB
HER2/neu/M (CB-11)	Breast ca (S)	BioGenex	1:20	CA	ABC/DAB
CD15/M (Leu M1)	LN (S)	Ventana	1:20	CA	ABC/DAB
CD20/M (L26)	LN (S)	DakoCytomation	1:750	CA	ABC/DAB
CD3/P (CMC365)	LN (S)	Cell Marque	1:300	CA	ABC/DAB
CD68/M (KP1)	LN (S)	DakoCytomation	1:400	CA	ABC/DAB
E-cadherin/M (4A2C7)	Bladder; thyroid ca (F)	Invitrogen/Zymed	1:100	CA	ABC/DAB

ABC, avidin-biotin complex; ca, carcinoma; CA, citraconic anhydride; CEA, carcinoembryonic antigen; CK, cytokeratin; DAB, diaminobenzidine; ER, estrogen receptor; F, fresh tissue tested within 1 month; GRP, glucose-regulated protein; LN, lymph node; M, monoclonal; P, polyclonal; Rb, retinoblastoma; S, frozen tissue stored longer than 1 month.
* LNCaP, MCF-7, and C42B are fresh prepared cell lines.
† Biocare Medical, Concord, CA; BioGenex Laboratories, San Ramon, CA; BD Biosciences, San Diego, CA; Cell Marque, Sacramento, CA; Chemicon International, Temecula, CA; DakoCytomation, Carpinteria, CA; EMD Calbiochem, Oncogene, San Diego, CA; Invitrogen/Zymed Laboratories, Carlsbad, CA; Lab Vision, Fremont, CA; Ventana Medical Systems, Tucson, AZ.

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Cell Lines

LNCaP and C42B were grown in RPMI 1640; MCF-7 cells in Dulbecco modified eagle's medium (Invitrogen, Carlsbad, CA) with 50 U/mL of penicillin, 50 U/mL of streptomycin, and 10% fetal calf serum (Mediatech, Herndon, VA). All cell lines were maintained in a humidified incubator at 5% carbon dioxide and 37°C. Cells were harvested routinely. After washing in 0.1 mol/L of phosphate-buffered saline (PBS; pH 7.2), Cytospin slide preparations were processed by using a Cytospin device (Hettich, Tuttlingen, Germany). All remaining cells were centrifuged at 1,000 rpm for 5 minutes. Cell pellets were processed in 2 parts as paired specimens: one part was quickly frozen in liquid nitrogen, then stored at –70°C for Western blot analysis, if required; a second part was fixed in 10% NBF overnight and then embedded routinely in paraffin as described for human tissue samples.

Fixation of Frozen Tissue Sections (Cytospin Slides Included)

Frozen tissue sections (Cytospin slides are included with frozen tissue sections in the following text) were air dried for 10 minutes and fixed by the following 6 protocols: (1) acetone for 10 minutes at room temperature, stored at –20°C before use; (2) ethanol (90%) for 10 minutes at room temperature, stored at –20°C before use; (3) 10% NBF for 30 minutes at room temperature, washed by PBS (pH 7.4), stored in PBS at room temperature before use; (4) 10% NBF containing 25 mmol/L of calcium chloride for 30 minutes at room temperature, washed by PBS, stored in PBS at room temperature before use; (5) 10% NBF at room temperature overnight; and (6) 10% NBF containing 25 mmol/L of calcium chloride at room temperature overnight.

Antigen Retrieval

A microwave boiling AR method was used in a plastic pressure cooker for 15 minutes in a solution of 0.05% citraconic anhydride at pH 7.4 (Sigma Chemical, St Louis, MO) for FFPE sections and the frozen tissue sections fixed by NBF or NBF + calcium chloride. In each case, AR was omitted on 1 tissue slide as a control experiment. For selected markers, other AR solutions were evaluated to replace the citraconic anhydride if the immunohistochemical staining results were poor (Table 1).

Immunohistochemical Analysis

Before the staining protocol, all frozen tissue sections were set at room temperature for 20 minutes and washed with PBS. FFPE tissue sections were processed routinely with deparaffinization, and methanol–hydrogen peroxide was used to block endogenous enzyme. Normal mouse or goat serum was used to block nonspecific binding reactions as appropriate. A total of 26 primary antibodies were used for this study, as listed in Table 1.

The Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used for immunohistochemical staining following the manufacturer's instructions. Other detection systems were selectively used when the immunohistochemical staining results were not satisfactory (Table 1) according to our routine protocols that have been used in recent years. To avoid potential variations among different batches of immunohistochemical staining procedures, all slides tested with each individual antibody were completed in a single "run" for more accurate comparison.

Serial titrations to establish optimal concentrations for each of the 26 primary antibodies tested were based on FFPE tissue sections with overnight incubation at room temperature (Table 1). According to the literature and our experience, the same concentration of each primary antibody (as used for FFPE sections) with an incubation time of 2 hours was used for frozen tissue sections to minimize nonspecific background staining and achieve an optimal signal-to-noise result.[5]

A comparative study was performed on acetone-fixed frozen tissue sections in a side-by-side manner to compare the immunohistochemical intensity between 2-hour and overnight primary incubations in order to validate the concentrations and the protocol (data not shown). For most markers, the link or secondary antibody (biotinylated antimouse or antirabbit immunoglobulin) and label (avidin-biotin complex) were incubated for 45 and 30 minutes, respectively. All incubations were performed at room temperature. A wash step with PBS for 10 minutes was carried out between each step of immunohistochemical staining. The chromogen was 3,3′-diaminobenzidine tetrahydrochloride. Slides were counterstained with Mayer hematoxylin.

Slides with a known positive reaction for each tested antibody were used as positive control slides, and slides with AR treatment were used as negative control slides by replacing primary antibodies with PBS. To confirm the staining results, all tests were carried out in triplicate.

Evaluation of Immunohistochemical Staining Results

Immunostained slides were evaluated by 2 observers (S.-R.S. and C.R.T.) independently in a blinded manner by light microscopy. The intensity of positive immunostaining was graded as 3+, 2+, 1+, or – for strong, moderate, weak, or negative, respectively; ± was used to represent focal or questionable weakly positive cells in tissue sections.

Western Blot Analysis

Cell lysates from LNCaP and C42B cells were prepared by lysing in 1 mL ice-cold radioimmunoprecipitation assay buffer. Equal measured amounts of total protein from each sample were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a 7.5% tris(hydroxymethyl)aminomethane hydrochloride gel (Bio-Rad Laboratories, Hercules, CA).

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Following electrophoresis, the proteins were transferred to a pure nitrocellulose membrane (Bio-Rad Laboratories). The membrane was then incubated in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) followed by overnight incubation with primary rabbit polyclonal anti–GRP 78 antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), primary monoclonal antisurvivin antibody (D-8, 1: 200 dilution; Santa Cruz Biotechnology), and primary monoclonal anti-p21 or polyclonal anti-p27 antibody (p21, 1:100 dilution; EMD Calbiochem, San Diego, CA; p27, C-19, 1:100 dilution; Santa Cruz Biotechnology). Signal detection was accomplished using Alexa Fluor 680 goat antirabbit antibody (Molecular Probes, Eugene, OR), IRDye 800 rabbit antimouse antitbody (Rockland Immunochemicals, Gilbertsville, PA), and subsequent scanning of the membrane by the Odyssey Infrared Imager (model 9120, Li-Cor Biosciences). All bands from Western blot analysis were quantified for protein expression with Odyssey Infrared Imaging Software (Li-Cor Biosciences) to assess integrated intensity (pixel volume) as a measure of absorbance. Band density was represented as the ratio of average band intensity of each sample to the average band intensity of the corresponding β-actin control band.

Results

Overall, FFPE tissue sections with AR treatment gave the best results in immunohistochemical staining for most antibodies tested, as judged by intensity of staining reaction, good morphologic features, and clean background **Table 2**, **Image 1**, and **Figure 1**. All negative control tissue and cell slides showed reliable negative results.

The comparison of immunohistochemical staining intensity alone between acetone-fixed and NBF-fixed frozen tissue sections revealed findings that were somewhat unexpected. More than half of the antibodies (16/26 [62%]) showed identical immunohistochemical staining intensity between the 2 groups. Among the remaining antibodies, 8 (31%) showed better immunohistochemical signals for NBF-fixed frozen tissue sections. Only 2 antibodies gave better immunohistochemical staining results for acetone-fixed frozen tissue sections. Frozen tissue sections fixed in NBF + calcium chloride solution yielded similar results to those obtained in frozen tissue sections fixed in NBF alone. With regard to ethanol fixation, more than half (16/26 [62%]) of the antibodies tested showed identical immunohistochemical staining intensity

Table 2
Comparison of Immunohistochemical Staining Results*

	Frozen Section Fixed in							
	Acetone or Ethanol (10 min)		NBF (30 min)		NBF (Overnight)		FFPE Section	
Antibodies Tested	Acetone	Ethanol	w/o AR	w/AR	w/o AR	w/AR	w/o AR	w/AR
ER	2+	2+	2+	3+	–	3+	–	3+
MIB-1	3+	3+	2+	3+	±	3+	–	3+
p53	3+	3+	3+	3+	2+	3+	1+~2+	3+
p27	–	±	–	3+	–	3+	2+	3+
Rb protein	2+	±	2+	3+	±	3+	–	3+
p21	±	±	2+	3+	1+	3+	–	3+
Pan-keratin	3+	2+	3+	3+	1+	3+	–	3+
S-100	–	–	1+	3+	–	3+	±	3+
Vimentin	3+	2+	2+	3+	1+	3+	1+	3+
CK7	3+	2+	3+	3+	2+	3+	1+	3+
CK20	3+	3+	3+	3+	2+	3+	–	3+
Desmin	3+	3+	2+	3+	2+	3+	2+	3+
Actin	3+	3+	3+	3+	2+	3+	2+	3+
Factor VIII antigen	3+	3+	3+	3+	3+	3+	–	3+
CEA	3+	3+	2+	3+	2+	3+	1+	3+
GRP 78	±	±	2+	3+	±	3+	±	3+
Melanosome Melan A	3+	1+	2+	3+	2+	3+	±	3+
Survivin	2+	2+	2+	3+	±	3+	–	3+
bcl-2 Oncoprotein	3+	1+	2+	3+	1+	3+	–	3+
CD45	3+	3+	3+	2+	2+	2+	2+	3+
HER2/neu	3+	2+	2+	3+	2+	3+	1+	3+
CD15	–	2+	3+	2+	2+	3+	±	3+
CD20	3+	3+	3+	3+	2+	3+	1+	3+
CD3	3+	3+	2+	3+	–	3+	–	3+
CD68	3+	3+	2+	1+	±	2+	–	3+
E-cadherin	3+	1+	2+	3+	1+	3+	–	3+

AR, antigen retrieval; CEA, carcinoembryonic antigen; CK, cytokeratin; ER, estrogen receptor; FFPE, formalin-fixed, paraffin-embedded; GRP, glucose-regulated protein; NBF, neutral buffered formalin; Rb, retinoblastoma; w/AR, use of the AR pretreatment before the immunohistochemical staining procedure; w/o AR, without use of the AR pretreatment.

* Scoring was as follows: 1+, weak; 2+, moderate; 3+, strong; –, negative; ±, focal or questionable weakly positive.

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between acetone- and ethanol-fixed frozen tissue sections. For acetone-fixed frozen tissue sections, approximately one third (8/26 [31%]) of the antibodies showed better immunohistochemical results than were obtained by ethanol fixation. The remaining 2 antibodies showed better immunohistochemical results for ethanol-fixed frozen tissue sections. The findings are summarized in Table 2 and illustrated in Image 1 and Figure 1.

It seems that most cytoplasmic proteins (10/13) showed comparable immunohistochemical signals between acetone- and NBF-fixed frozen tissue sections. For nuclear proteins, NBF-fixed frozen tissue sections gave better immunohistochemical signals than were obtained by acetone-fixed sections. In particular, the p21-stained nuclear staining picture showed a granular appearance or a surrounding nuclear staining pattern **Image 2**. For cell surface proteins (CD markers), acetone-fixed frozen tissue sections showed better results for antibodies such as CD68.

A comparison of morphologic features for frozen tissue sections demonstrated apparently better cell and tissue morphologic features achieved by NBF-fixed frozen tissue sections (Image 1). Furthermore, NBF-fixed frozen tissue sections always gave less nonspecific immunohistochemical background staining than that observed in acetone- or ethanol-fixed sections, particularly after AR treatment **Image 3**.

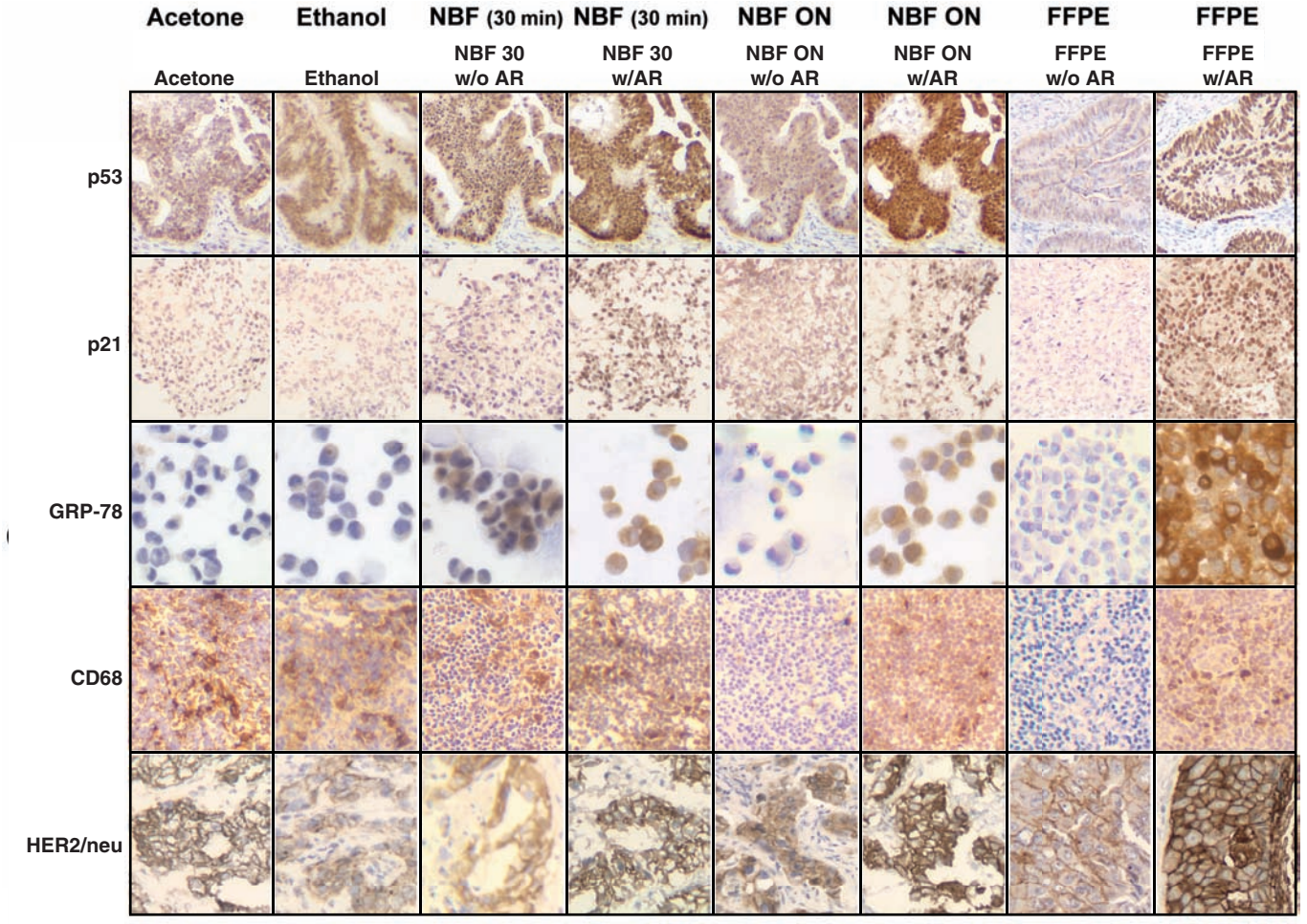

Image 1 Comparison of immunohistochemical staining intensity among various protocols of fixation, antigen-retrieval (AR) pretreatment for frozen and formalin-fixed, paraffin-embedded (FFPE) cell and tissue sections. Five markers are selected as examples from Table 2: p53-stained colon cancer tissue (first row); p21-stained bladder cancer tissue (second row); GRP-78–stained cell line C42B (third row); CD68-stained lymph node tissue (fourth row); and HER2-stained breast cancer tissue (fifth row). In general, neutral buffered formalin (NBF)-fixed frozen cell/tissue with AR treatment showed identical or stronger immunohistochemical staining intensity compared with that obtained by acetone- or ethanol-fixed cell/tissue, except with CD68. FFPE cell and tissue sections yield the strongest immunohistochemical signals and the best morphologic features consistently. NBF 30, NBF for 30 min; ON, overnight; w/AR, use of the AR pretreatment before the immunohistochemical staining procedure; w/o AR, without use of the AR pretreatment (×200).

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The Western blot assay showed definite protein bands for 4 markers, p21, p27, GRP 78, and survivin **Image 4** that reflected the immunohistochemically positive results for the markers obtained in the NBF-fixed tissue sections but negative or weakly positive staining reactions for frozen tissue sections fixed by acetone or ethanol (Image 4 vs Image 1 and Table 2).

Discussion

Based on this study, it is clear that the traditional concept that the gold standard for immunohistochemical analysis is achieved by using acetone- or ethanol-fixed frozen tissue section is not entirely correct. Many proteins (antigens or epitopes thereof) were observed to be lost partially or completely following acetone- or ethanol-based fixation of frozen sections

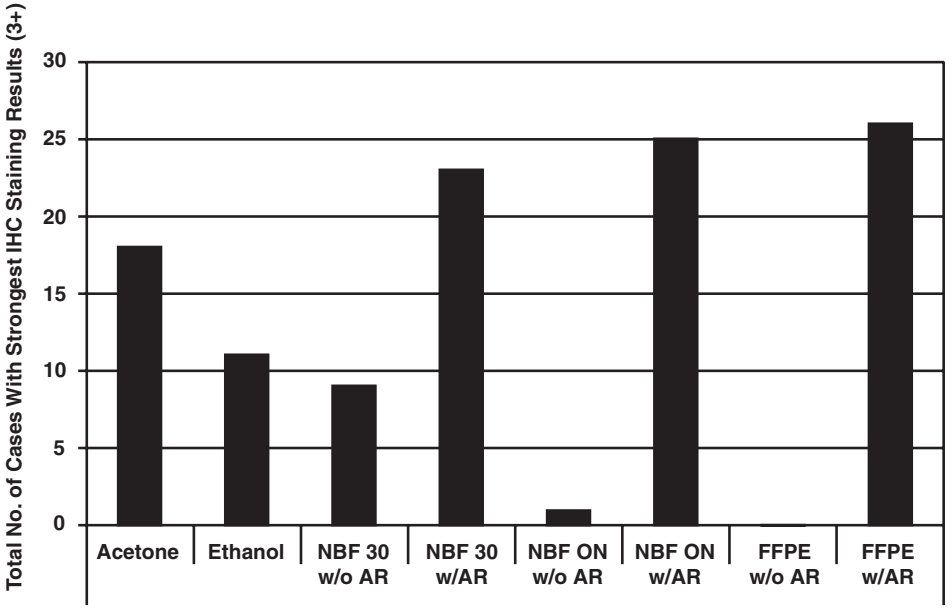

Figure 1 Comparison of immunohistochemical staining intensity among various protocols of fixation, processing, and antigen retrieval (AR) immunohistochemical (IHC) analysis as indicated in Table 2. The strongest immunohistochemical staining intensity was achieved in formalin-fixed, paraffin-embedded (FFPE) tissue sections after AR treatment. NBF 30, neutral buffered formalin for 30 min; ON, overnight; w/AR, use of the AR pretreatment before the immunohistochemical staining procedure; w/o AR, without use of the AR pretreatment.

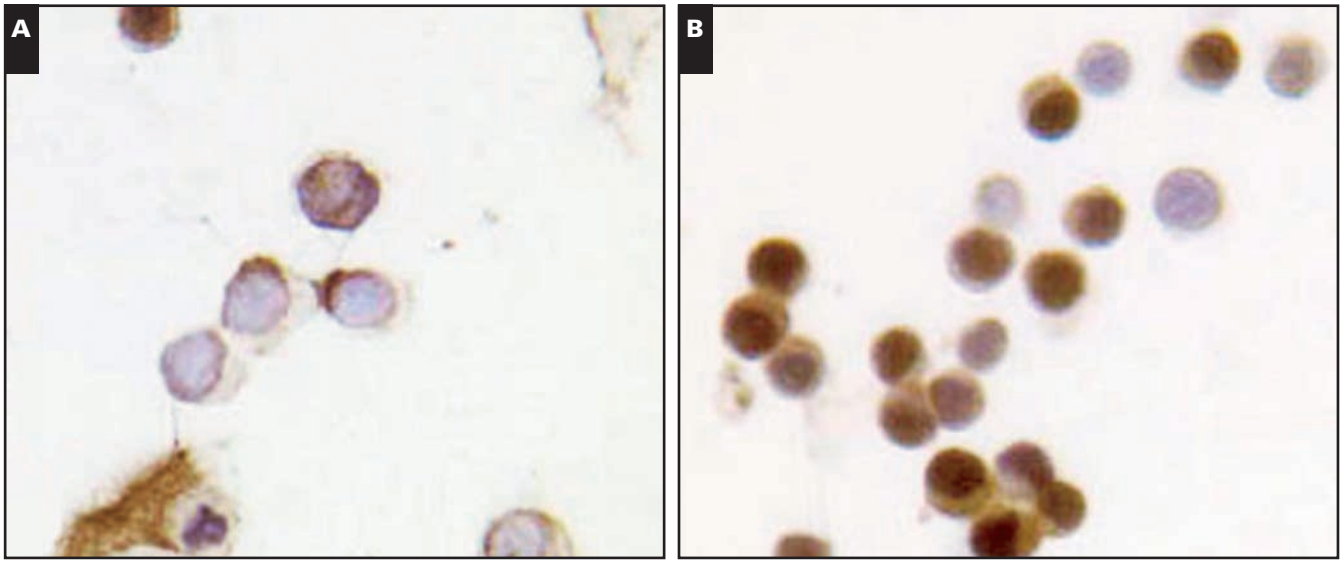

Image 2 Comparison of p21 immunohistochemical staining results using fresh cell line MCF-7. **A**, Acetone-fixed cells showing an irregular positive staining pattern indicating dislocalized p21 protein from nuclei to cytoplasm and outside of cells (×400). **B**, Neutral buffered formalin–fixed cells with the use of antigen-retrieval treatment before immunohistochemical staining showing an intact nuclear p21 staining pattern (×400).

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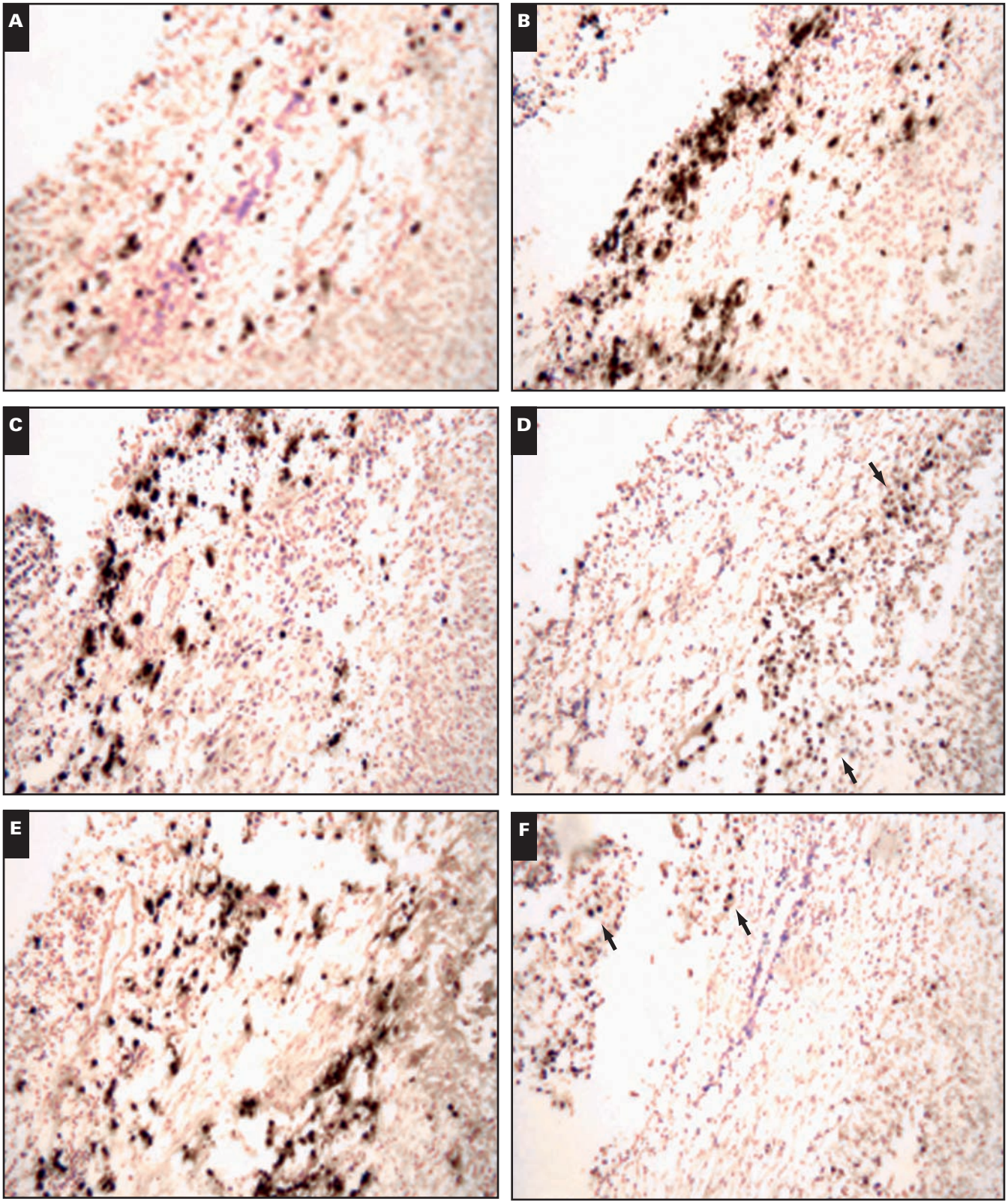

Image 3 Comparison of nonspecific background immunohistochemical staining results among various fixations of frozen tissue sections and immunohistochemical antigen-retrieval (AR) staining protocols. Human bladder cancer tissue samples were used for the p21 staining procedure. Significant, strong, nonspecific background staining results can be found in acetone-fixed (**A**), ethanol-fixed (**B**), neutral buffered formalin (NBF)-fixed (for 30 min; **C**), and NBF-fixed (overnight; **E**) samples showing irregular large dots that stained positively. In contrast, the same kinds of NBF-fixed frozen tissue sections after AR treatment before immunohistochemical staining (**D** and **F**) showed clear background. Arrows indicate the p21+ nuclear staining results (**A**-**F**, ×100).

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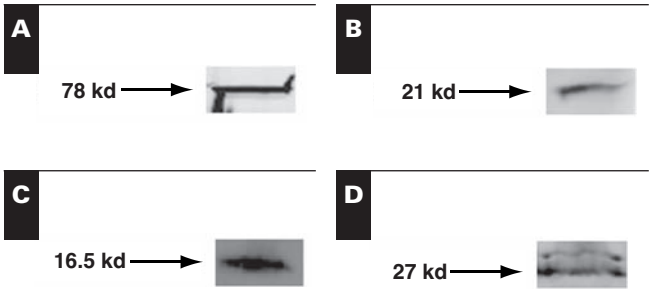

Image 4 Western blot assay showing 4 protein bands for demonstration of GRP 78 (**A**), p21 (**B**), survivin (**C**), and p27 (**D**) to demonstrate that the positive immunohistochemical staining results found in neutral buffered formalin–fixed samples with use of these 4 primary antibodies for their correlated proteins are true. **A** and **C**, C42B cells; **B** and **D**, MCF-7 cells.

(Table 2, Images 1 and 2, and Figure 1) in comparison with FFPE preparations of the same tissues. Although the extent of these findings is unexpected, it should not be wholly surprising. Loss of immunoreactivity after "coagulant" fixatives (eg, ethanol and acetone) has been documented previously.[6] Also, as described, Yamashita and Okada[4] demonstrated that half of the commonly used antibodies they tested by immunohistochemical analysis showed significantly reduced signals for acetone-fixed fresh tissue sections when compared with tissue fixed in formaldehyde.

It has been recognized that low-molecular-weight proteins (antigens) and lipoproteins are readily extracted by coagulant fixatives.[6] In 1 study, about 13% of total protein was lost following acetone fixation.[7] In the present study, comparison of formalin-fixed cells showing distinct, intense nuclear staining for p21 with acetone- or ethanol-fixed fresh cell smears showing characteristic morphologic features but a low-intensity p21 reaction may be interpreted to indicate that "some" p21 protein has been extracted from the nuclei by acetone or ethanol (Image 2). Although the mechanism of tissue fixation is not clear, it has been proposed that all coagulating fixatives are dehydrants that disrupt the protein conformation through removal of free water from tissue resulting in denaturation and loss of function.[7]

In contrast with coagulating fixatives, formaldehyde is a cross-linking fixative characterized by fixing proteins in situ through formation of extensive intramolecular and intermolecular covalent cross-links.[8,9] Numerous studies have demonstrated that most of the proteins in tissues are preserved quite well by formaldehyde fixation, as demonstrated by an abundance of immunohistochemical studies using the AR treatment and recent proteomic studies of proteins extracted from FFPE tissue sections.[10-14] In a recent collaborative study, we were able to demonstrate more than 3,000 different protein signatures by mass spectrometry in an extract of FFPE human tissue prepared by using a heat-induced AR technique.[13] These studies suggest that the extensive intramolecular and intermolecular cross-links of tissue proteins induced by formalin in fact provide a reliable way to stabilize proteins in tissue and to protect against their loss; the simple boiling AR method serves to recover or restore the structure of many of these formalin-fixed proteins, at least to a degree sufficient for satisfactory immunohistochemical staining.

The worldwide application of the AR technique for immunohistochemical staining on FFPE tissue sections has in effect created "pre" and "post" AR eras in the literature,[2,5] but lingering doubts remain as to the structural integrity of "fixed and retrieved" protein. From the present study (Table 2, Images 1-3, and Figure 1), it is apparent that the FFPE tissue sections give a strong immunohistochemical signal, with the bonus of superior morphologic features and clean background. In another study, Shidham and coworkers[15] reported a comparative study using scraped cell smears from the fresh-cut surface of 45 unfixed fresh tumor specimens to identify the most suitable method of smear preparation and fixation by immunohistochemical staining. Based on immunohistochemical staining of FFPE tissue sections with heat-induced AR treatment used as the standard positive control (gold standard), they compared staining intensity, scored semiquantitatively, among 3 groups of wet 95% ethanol-fixed and air-dried saline rehydrated smears, fixed in alcoholic formalin or 95% ethanol with 5% acetic acid. Their conclusion also demonstrated that FFPE tissue sections with use of AR showed the best immunohistochemical staining results,[15] despite the fact that a few proteins are not satisfactory after current AR treatment and need further development of the AR technique.

More and more articles in the recent literature reporting immunohistochemical studies have been based on the use of FFPE tissue sections only, based on the presumption, perhaps, that validation by frozen section analysis is unnecessary. In essence, the FFPE tissue section with an optimal AR treatment has quietly been accepted as the gold standard for many immunohistochemical studies. It has long been argued that, in theory, frozen tissue sections should yield better preservation of natural proteins absent the chemical modification intrinsic to fixatives such as formalin. In practice, however, stored frozen tissue sections may not maintain satisfactory localization or preservation of proteins for immunohistochemical staining or for the study of extracted proteins, and, certainly, morphologic features are compromised. Several factors may contribute to possible losses in frozen sections, including degradation, autolysis, and diffusion occurring before effective freezing; inadequate or inconsistent low temperature storage conditions; and tissue heating by compression during the preparation of frozen tissue sections.

Nevertheless, a critical issue must be addressed for the proteins (antigens) showing negative immunohistochemical staining results when using acetone- or ethanol-fixed cell or tissue sections but positive staining results when using NBF-fixed samples with AR treatment. One potential for these variable immunohistochemical results needs to be validated by other objective

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methods, otherwise, as pointed out by Wick and Mills,[16] "there is a real risk that artifacts may become 'facts.' " Western blot analysis was performed in the present study (Image 4) to demonstrate 4 proteins that, after AR treatment on NBF-fixed samples, showed strong positive immunohistochemical signals that are not artifacts.

A further observation of interest was that all NBF-fixed frozen sections and FFPE tissue sections, each with AR, showed dramatically reduced nonspecific background staining (Image 3). The underlying mechanism is unclear. It may be postulated that AR treatment may alter the overall electrostatic charge of the tissue leading to reduced nonspecific binding, but other possibilities exist.[17] In one direct practical application, the advantage of a cleaner background achieved by formaldehyde fixation of fresh cell samples has been exploited for the detection of disseminated tumor cells in bone marrow by immunohistochemical staining of Cytospin slides fixed in buffered formalin or paraformaldehyde for 10 minutes, showing satisfactory results for morphologic features and immunohistochemical signals.[18] From Table 2, it is obvious that 9 markers (including 3 antibodies to keratin) yielded strong immunohistochemical signals after NBF fixation for 30 minutes without AR. Therefore, 10-minute, NBF-fixed cell smear slides will be used as a satisfactory standard protocol for immunohistochemical detection of rare disseminated cancer cells in bone marrow or peripheral blood even without AR. Swerts and coworkers[18] emphasized that formaldehyde used as a Cytospin fixative should be free of methanol. In our hands, acetone-fixed bone marrow slides gave poor morphologic features and abundant nonspecific background staining, resulting in difficult interpretation when detecting cancer cells as a rare event among sheets of normal cells.

In addition, NBF-fixed frozen tissue sections may be used as a simple method to find the degree of sensitivity of proteins to formalin fixation by using frozen tissue sections fixed in NBF for variable time schedules as shown in Table 2. It seems that the results from frozen tissue sections fixed in NBF overnight may be set as cutoff points. Table 2 indicates that estrogen receptor, S-100, MIB-1, GRP 78, p21, p27, retinoblastoma protein, CD3, and CD68 may be categorized into formalin-sensitive proteins; factor VIII and carcinoembryonic antigen as formalin-resistant proteins; and others as a moderate type of proteins.

Our data do not support the traditional use of acetone-fixed frozen tissue sections as the gold standard for immunohistochemical staining. In examining any new antigen, it would be prudent to use a combination of acetone-fixed frozen sections and NBF fixation. FFPE tissue sections may serve as the standard for most antigens for immunohistochemical staining.

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