Journal of Histochemistry & Cytochemistry 59(1) 13–32

DOI: 10.1369/jhc.2010.957191
http://jhc.sagepub.com
SAGE

Antigen Retrieval Immunohistochemistry: Review and Future Prospects in Research and Diagnosis over Two Decades

Shan-Rong Shi, Yan Shi, and Clive R. Taylor
Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, California (SRS, CRT) and Department of Pathology, New York University, Langone Medical Center (YS).

Summary

As a review for the 20th anniversary of publishing the antigen retrieval (AR) technique in this journal, the authors intend briefly to summarize developments in AR-immunohistochemistry (IHC)–based research and diagnostics, with particular emphasis on current challenges and future research directions. Over the past 20 years, the efforts of many different investigators have coalesced in extending the AR approach to all areas of anatomic pathology diagnosis and research and further have led to AR-based protein extraction techniques and tissue-based proteomics. As a result, formalin-fixed paraffin-embedded (FFPE) archival tissue collections are now seen as a literal treasure of materials for clinical and translational research to an extent unimaginable just two decades ago. Further research in AR-IHC is likely to focus on tissue proteomics, developing a more efficient protocol for protein extraction from FFPE tissue based on the AR principle, and combining the proteomics approach with AR-IHC to establish a practical, sophisticated platform for identifying and using biomarkers in personalized medicine. (J Histochem Cytochem 59:13–32, 2011)

Keywords

antigen retrieval (AR), immunohistochemistry (IHC), formalin-fixed, paraffin-embedded (FFPE) tissue, quantitative immunohistochemistry (QIHC), quantifiable reference standards, protein extraction, proteomics, cytopathology

Just two decades have passed since the first article on antigen retrieval (AR) was published in 1991 (Shi et al. 1991). This simple technique of boiling formalin-fixed paraffin-embedded (FFPE) tissue sections in water has played a major role in extending the reach and use of immunohistochemistry (IHC) in FFPE tissues (Gown et al. 1993; Taylor and Cote 2005). One notable result is the effective division of all publications with respect to IHC for FFPE tissue sections into two eras: pre-AR and post-AR (Gown 2004; Taylor 2001), indicating AR as a milestone (Jagirdar 2008). Since the early 1950s, the *Journal of Histochemistry & Cytochemistry* has published numerous articles describing new and interesting techniques for morphologic examination of tissues, such as the enzyme-labeled IHC (Nakane and Pierce 1966), avidin-biotin detection system (Hsu et al. 1981), AR (Shi et al. 1991; Shi et al. 1992), tyramide signal amplification (Adams 1992), and so on. All of these valuable developments have contributed to clinical and basic biomedical research projects worldwide.

A number of factors contribute to the major impact that AR has had on diagnostic pathology (Boon and Kok 1995; Dabbs 2008; Jagirdar 2008; Hawes et al. 2010; Taylor et al. 2010). First, for more than one hundred years, FFPE tissues have served as the standard tissue preparation method in surgical pathology, providing the basis for most of the criteria for pathological diagnosis in "routine" hematoxylin and eosin–stained FFPE tissue sections. AR critically facilitated the use of FFPE tissues for IHC, retaining the use of existing morphologic criteria. Second, as a direct result of this extended capability, the value of archival FFPE tissue blocks, accompanied by known follow-up data, was enormously enhanced,

Received for publication July 27, 2010; accepted October 21, 2010

Corresponding Author:
Shan-Rong Shi, Department of Pathology, University of Southern California Keck School of Medicine, 2011 Zonal Avenue, HMR 310A, Los Angeles, CA 90033.
E-mail: sshi@usc.edu

providing a valuable resource for translational clinical research and basic research that cannot easily be reproduced. Third, AR is a simple, inexpensive, and effective technique that leads to satisfactory IHC staining results in FFPE tissue for a great number of antibodies tested (Shi et al. 1995; Shi et al. 1997; Taylor and Cote 2005; Yamashita 2007; Shi and Taylor 2010a; Taylor et al. 2010). Finally, and by no means least, there is a growing body of recent literature with respect to use of AR methods for extraction of molecules (DNA, RNA, and proteins) from FFPE tissues that has further extended the utility of archival tissues and promises to allow the combination of proteomics and IHC in a true molecular morphology approach. So effective have these AR-based approaches become that investigators are now actively exploring the possible advantages of FFPE tissues in terms of preservation of both morphology and molecules in cell/tissue samples, in comparison to other methods of sample preparation (Frank et al. 1996; Masuda et al. 1999; Sato et al. 2001; Shi et al. 2002; Shi et al. 2006; Palmer-Toy et al. 2005; Becker et al. 2007; Guo et al. 2007; Jiang et al. 2007; Addis et al. 2009; Fowler CB et al. 2010; Shi and Taylor 2010c).

In concept and development, the primary goal of the AR methods was always to meet the needs of clinical practice, specifically to facilitate the performance of IHC on FFPE tissues. There was a considerable prior literature describing the use of IHC on FFPE tissues, with many attempts to improve the quality of results (Taylor and Burns 1974; Taylor and Mason 1974; Huang 1975; Hausen and Dreyer 1982; Taylor 1979; Kitamoto et al. 1987; Abbondanzo et al. 1991). Many of the active pioneers were practicing pathologists, acutely aware of the need to enhance the capabilities of IHC on FFPE tissues, so as to retain the key morphologic features that form the basis of diagnostic histopathology (Taylor 1980; Taylor and Kledzik 1981; Pinkus 1982; Colvin et al. 1995; Taylor and Cote 2005; Shi and Taylor 2010f). Although the AR technique is simple in concept and execution, development was difficult, in large part, because the notion of heating tissues to improve "antigenicity" was counterintuitive. As the author (Shi) began to formulate the idea in the 1980s, there were a number of practical and theoretical issues to be addressed. A key scientific question was whether fixation in formalin modified the structure of antigens in a reversible or irreversible manner. To be more specific, was there any theoretical or prior scientific evidence that the effects of formalin fixation on proteins could be reversed, and if reversed, was the structure of protein restored to a sufficient degree for recovery of antigenicity? With these key questions in mind, Shi spent many days and nights in 1988 searching the chemical literature under difficult conditions, prior to the increased efficiency of such searches that is afforded today by the Internet and online databases. The answer was finally found in a series of studies of the chemical reactions between protein and formalin, published in the 1940s (Fraenkel-Conrat et al. 1947; Fraenkel-Conrat and Olcott 1948a, 1948b). These studies indicated that cross-linkages between formalin and protein could be disrupted by heating above 100C or by strong alkaline treatment. With this knowledge of high temperature heating as a potential retrieval approach, the heat-induced AR technique was developed in 1991 (Shi and Taylor 2010d).

Today, 20 years on, the AR technique is widely, almost universally, used in surgical pathology, including veterinary pathology (Ramos-Vara 2005), in all morphology-based sciences, and in pharmacology drug-related research, with thousands of original articles published worldwide, together with more than one dozen review articles, with those by Yamashita and D'Amico being most recent (D'Amico et al. 2009; Yamashita 2007). Two books edited by our group summarize almost all data pertaining to further technical development and application of AR (Shi, Gu, Taylor 2000; Shi and Taylor 2010b).

The present review is focused on several critical issues, with an emphasis on current challenges and potentially productive directions for future study. Five areas of research have been identified within these parameters: (1) the role of AR in improved standardization of IHC, (2) AR as a component of cell/tissue sample preparation, (3) the mechanism of AR, (4) extraction of nucleic acids and proteins from FFPE tissue sections for genomic and proteomic analysis, and (5) combining proteomics and IHC for quantitative analysis of defined cell populations (molecular morphology) in research and diagnosis. These five themes are woven throughout the following discussion.

Extending the Application of AR-IHC

To date, AR has been applied predominantly to archival "paraffin blocks" for IHC in diagnostic surgical pathology as a routine procedure. There are, in addition, many other adaptations of the AR method: for improved IHC staining of plastic-embedded tissue samples both by light and electron microscopy, as a blocking procedure to avoid cross-antigen/antibody reaction during multiple IHC staining procedures, for enhancement of DNA/RNA in situ hybridization in FFPE materials, for in situ end-labeling (terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL]) of apoptotic cells in FFPE tissue sections, and in flow cytometry to achieve stronger positive signals while reducing nonspecific background noise (Shi, Cote, Shi, et al 2000). Application of AR to cytopathology, frozen sections, and immunofluorescence methods will be reviewed in more detail. Major applications of AR technique or its principles are summarized in Table 1.

Cytopathology. Application of IHC (or ICC, signifying immunocytochemistry) in cytopathology has lagged behind use in histopathology, in part because of differences in cell sample preparation, which is quite different from that used

Table 1. Major Applications of Antigen Retrieval Technique and Principle

Areas of Application of AR	Application of AR Technique and/or Principle	Reference
IEM	AR pretreatment of routine processed Epon-embedded tissue ultra-thin sections after etching the grids by solutions[a] to achieve satisfactory positive results or directly heating the grid, followed by some washing procedures, including 50 mM NH_4Cl and 1% Tween 20	Stirling and Graff 1995; Wilson et al. 1996
ISH	High-temperature heating FFPE tissue sections prior to ISH to achieve satisfactory results	Sibony et al. 1995; Lan et al. 1996; McMahon and McQuaid 1996
TUNEL	Optimal heating time such as 1 min to improve the signal	Strater et al. 1995; Lucassen et al. 2000
Multiple IHC staining procedure	Adding a microwave heating AR procedure (10 min) between each run of IHC staining procedure to block the cross-reaction by denaturing bound antibody molecules from the previous run	Lan et al. 1995
Human temporal bone collections	Combining sodium hydroxide–methanol and heating AR treatment provides an effective approach for IHC used in celloidin-embedded temporal bone sections. This method is also used for plastic-embedded tissue sections, including IEM	Shi et al. 1992; Shi, Cote, Taylor 2000
Immunofluorescence	To enhance intensity and reduce autofluorescence	D'Ambra-Cabry et al. 1995
Cytopathology	Boiling AR pretreatment for archival Pap smear slides to achieve satisfactory IHC staining for MIB-1 used for fine-tuning diagnoses in cervical cytology. Formalin postfixed air-dried smears	Boon et al. 1994; Boon et al. 1995; Boon et al. 2000 Fulciniti et al. 2008; Chivukula and Dabbs 2010
FCM	Enzyme digestion followed by heating AR treatment was adopted to achieve enhancement of FCM on FFPE tissue	Redkar and Krishan 1999
Floating vibratome section	Microwave boiling vibratome section to achieve IHC staining results that further extended the use for whole-mount tissue specimens	Evers and Uylings 1994, 2000; Shiurba et al. 1998
En bloc tissue	AR heating 4% paraformaldehyde-fixed animal brain or testis tissue blocks to enhance immunoreactivity for most antibodies tested	Ino. 2003
Frozen tissue section	Aldehyde-fixed frozen tissue section with use of AR treatment to achieve both excellent morphology and IHC staining result	Yamashita and Okada 2005a; Shi et al. 2008
DNA extraction from FFPE tissue sections	Boiling AR pretreatment prior to DNA extraction to replace enzyme treatment for improved results of DNA extraction	Frank et al. 1996; Coombs et al. 1999; Shi et al. 2002; Shi et al. 2004
RNA extraction from FFPE tissue sections	Heating treatment prior to RNA extraction to recover fixative-induced modification or to replace enzyme treatment for improved results of RNA extraction	Masuda et al. 1999; Shi and Taylor 2010c
Protein extraction from FFPE tissue sections	Boiling AR pretreatment with AR solution, including 2% SDS and/or other chemicals, to improve efficiency of protein extraction from FFPE tissue sections to replace enzyme digestion. Further development by combining elevated hydrostatic pressure may increase extraction to 80% to 95% of proteins in FFPE tissue sections.	Ikeda et al. 1998; Shi et al. 2006; Fowler CB et al. 2007; Fowler CB et al. 2008; Fowler CB et al. 2010
IMS	Boiling AR pretreatment is being adopted to achieve satisfactory results of IMS in recent years. On the basis of comparing different AR solutions, Gustafsson et al. (2010) summarized that the citrate acid AR method is an important step in being able to fully analyze the proteome for FFPE tissue.	Groseclose et al. 2008; Ronci et al. 2008; Gustafsson et al. 2010

AR = antigen retrieval; FFPE = formalin-fixed paraffin-embedded; IEM = immunoelectron microscopy; ISH = in situ hybridization; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling; FCM = flow cytometry; IMS = imaging mass spectrometry.

[a]Ten percent fresh saturated solution of sodium ethoxide diluted with anhydrous ethanol for 2 min or with a saturated aqueous solution of sodium metaperiodate for 1 hr.

for FFPE tissues in surgical pathology. In cytopathology, the cell sample is limited to a small amount that allows for only a few "smear" or imprint slides for cytologic evaluation to make a diagnosis. This circumstance alone renders it difficult, if not impossible, to undertake a panel of IHC (or ICC) stains, as is frequently used in histopathology. In a minority of instances, when a larger amount of cell sample is obtained, it is possible to use the cell block technique, which does allow for the cutting of serial sections for a panel of IHC stains. On the basis of these conditions, L. J. Fowler and Lachar (2008) highlighted the challenges that exist in application of ICC to cytopathology. One of the major issues is lack of proper control samples. Another problem is the frequent use of inappropriate antibody concentrations due to a lack of appropriate cell samples for optimal titration studies; by default, dilutions established as suitable for FFPE sections are used, with resultant errors. Indeed, it should be emphasized that ICC controls for cytology specimens must be made from similarly prepared cell specimens for accurate comparison. Use of FFPE tissue section as a positive control for a cytology sample is not appropriate and is likely to result in misinterpretation. This problem represents a major practical obstacle because most hospital pathology laboratories lack the resources and expertise to establish appropriate cell line control systems.

AR Greatly Improves Sensitivity of ICC for Cytological Smears but Needs Standardization

Additional difficulties parallel those encountered in diagnostic surgical pathology and arise from the fact that protocols of cell sample preparation and fixation, used in cytopathology, also are not standardized across laboratories. More than a decade ago, Suthipintawong et al. (1996) performed a comparative study for 23 fixation protocols of cell preparations for ICC and reported that fixation in 0.1% formal saline overnight at 27C, followed by 10-min fixation in 100% ethanol, coupled with the use of microwave AR pretreatment, gave the best results. Gong et al. (2004) carefully compared ICC staining results of estrogen receptor (ER) between cell smears and corresponding tissue sections using the AR technique for several fixatives, including formaldehyde and Carnoy's fixative, and demonstrated that the use of the AR technique in cytological smears greatly improved ER immunodetectability in both formalin-fixed and Carnoy's-Pap smears, raising the final concordance rate with FFPE tissue sections from 31% (formalin fixed) and 29.4% (Carnoy's smears) without AR to 93% with AR. Although AR techniques increasingly are used in ICC for cytopathology, standardization remains a major problem (Dabbs 2010). Fetsch and Abati (2007) concluded that standardization of AR-ICC staining procedures is imperative for the optimal interpretation of HER2 because of variation in sample size, fixative, and preparation method. They obtained widely different results in a study of 54 FFPE cell block sections of metastatic breast cancer, using three primary antibodies to HER2 with one single heat-induced AR protocol (boiling sections in citric acid buffer of pH 6.0 for 20 min). Therefore, establishing an optimal AR protocol, based on the test battery approach for each primary antibody tested, is essential instead of using one single AR protocol (Shi and Taylor 2010e).

Current Trend: Formalin Postfixed Air-Dried Smears with AR Treatment Give Reliable ICC Results and Better Morphology

There is a current trend for a wider use of formalin fixation in cytopathology despite the fact that many of the existing sample preparation methods are fast and give good morphology (Suthipintawong et al. 1997; Shidham et al. 2000; Liu J and Farhood 2004; Fulciniti et al. 2008). Fulciniti et al. (2008) reported a study of ICC on fine-needle cytology samples comparing alcohol-wet-fixed and formalin postfixed air-dried cell smears and concluded that the formalin postfixed air-dried smears gave reliable ICC results with the use of AR treatment. In addition, the visual evaluation of results was easier in air-dried cells because of their larger, "well-flattened" morphology. Chivukula and Dabbs (2010) also advocated use of the formalin postfixed air-dried method and pointed out that certain antibodies such as S-100 protein, Hep Par 1, and gross cystic disease fluid protein–15 (GCDFP-15) gave false-negative results with alcohol-based fixatives due to leaching out of proteins. AR has also been used to enhance results in studies of archival Pap-stained cytological slides (Boon et al. 1994; Abendroth and Dabbs 1995). Subsequently, many articles have confirmed the value of AR methods for ICC staining of stored cytological slides (Liu J and Farhood 2004; Goel et al. 2005; Shtilbans et al. 2005; Shroyer et al. 2006; Yoshida et al. 2008).

Extending the Use of Cell Samples by Technical Development: Cell Block, Transfer, and Others

Cell block and cell transfer techniques are being used increasingly in the quest for improved standardization and wider application of ICC in cytopathology (Shi and Wasserman 2010). For the purpose of ICC, FFPE cell blocks are considered the most ideal samples. Cell blocks simulate surgical pathology samples. They can be handled and stored in a similar manner (Shin et al. 2007; Fowler LJ and Lachar 2008) and stained using AR and IHC protocols and antibody panels analogous to those used in surgical pathology, providing a stain quality that is superior to most ICC results on cytospins or smears (Fetsch and Abati 2001;

Fetsch et al. 2002). Therefore, this technique is highly recommended as the first choice for ICC analysis whenever enough cell samples are available (Fetsch and Abati 2001; Miller and Kubier 2002; DeLellis and Hoda 2006; Liu H et al. 2007; Shin et al. 2007). In cases where insufficient material is available for a cell block, the "Cytoscrape" method has been proposed, characterized by scraping off darkly stained tissue fragments from smeared slides (Verbeek et al. 1996; Kulkarni et al. 2000; Shi and Wasserman 2010). The potential advantages of a cell block not withstanding, there are many cases when there are just insufficient cells for a cell block preparation. In these circumstances, various recommendations allow multiple immunostaining on limited smears. In 1998, Dabbs and Wang reported "repeat ICC" for cytologic specimens of limited quantity. The principle of this method is that ICC can be performed more than once on the same cytologic specimen if the initial test is negative. Weintraub et al. (1990) reported a successful ICC study using three antibodies (AE1/AE3, CAM5.2, and leukocyte common antigen) on one single smear, an approach restricted by the number of antibodies one can apply and distinguish on a single slide.

The "cell transfer" technique provides another approach to performing multiple ICC tests on limited smears (Brown and Tao 1992; Dabbs and Wang 1998; Gong et al. 2005; Miller and Kubier 2002; Sherman et al. 1994). Because of the impressive results achieved by this approach, Miller and Kubier (2002) advocated the routine use of "non-adhesive" slides to facilitate use of the cell transfer technique when necessary. A further technical development, named the "multiplex-immunostain chip" (MI chip), has been reported to allow testing as many as 50 markers in one single tissue section (Furuya et al. 2004). The key point of this method is a unique 5-mm-thick silicon-rubber plate containing 50 small wells, each of which can be filled with various primary antibodies. The practical utility of this method remains to be evaluated.

It is generally recognized that increased application of the AR technique for ICC has improved comparability of findings with IHC on FFPE tissues and at the same time has increased the diagnostic utility of cytopathology. However, there is much room for improvement in both areas, particularly with respect to control and standardization.

Frozen section. Successful application of the heat-induced AR technique for frozen cell/tissue sections has recently been documented. Yamashita and Okada (2005a) examined the results of 22 antibodies comparing acetone-fixed and aldehyde-fixed frozen tissue sections. They reported that most antibodies showed stronger intensity of IHC for aldehyde-fixed frozen tissue sections, after the AR treatment, than obtained in acetone-fixed tissues. Of particular note, 11 (50%) antibodies gave negative IHC staining using acetone-fixed frozen tissue sections but yielded positive staining using aldehyde-fixed frozen tissue sections with AR. Similar findings on frozen cell/tissue sections have been documented by our group (Shi et al. 2008); more than half of the tested antibodies (16/26, 61.5%) showed identical IHC staining results between acetone-fixed and formalin-fixed tissue sections, 8 (30.8%) showed better IHC signals following formalin fixation and AR, and only 2 antibodies gave better IHC staining results for acetone-fixed frozen tissue sections. Most cytoplasmic proteins (10/13) showed comparable IHC signal between acetone and formalin-fixed tissue sections, but nuclear proteins gave better IHC signals following formalin fixation coupled with AR. In most cases, formalin fixation after AR yielded a stronger signal with less background and better morphology. In addition, we also found that some of the nuclear IHC staining patterns, such as p21 or p27, showed relocation of nuclear staining into the cytoplasmic or perinuclear area or even "leakage" from cells into the tissue space after acetone or alcohol fixation. It has long been recognized that some low molecular weight proteins and certain lipoproteins are readily extracted by coagulant fixatives (alcohol), and about 13% of total protein may be lost following acetone fixation (Larsson 1988; Eltoum et al. 2001). Another interesting observation is that the "nonspecific" background staining frequently found in frozen tissue sections during immunofluorescent (IF) or IHC staining is significantly reduced after AR treatment.

AR-IHC Immunostaining Results on FFPE Tissue Sections: "Gold Standard"

FFPE tissue sections, of course, provide superior morphology and now increasingly give equivalent or better IHC staining results. Thus, it is not surprising that AR-IHC immunostaining results have increasingly been accepted as the "gold standard" (Shidham et al. 2003; Dabbs 2008, 2010). However, when using FFPE tissues, it is prudent, whenever possible, to use independent objective biochemical methods, such as Western blot analysis, to validate antibody performance under different conditions of sample preparation. In this context, the antigens (proteins) used in Western blots should be prepared as far as possible in a manner that matches the processing of FFPE tissues. This independent validation is of particular importance for antibodies that show negative IHC staining when using coagulating fixatives for frozen cell/tissue sections but give positive staining results when using formalin-fixed samples with AR (Wick and Mills 2001).

IF staining. Recently, there has been an upsurge in the application of IF methods to FFPE tissues because of the development of new dyes, plus advantages of IF for quantitative measurement by image analysis (Rimm 2006) or for multiple IF labeling using spectral imaging microscopy (Taylor and Levenson 2006). In the past, IF was not commonly used with FFPE tissues because of inherent diffuse autofluorescence. Different strategies have been advanced

to combat this problem, including extraction of the autofluorescent constituents, chemical modification of the fluorochrome, photobleaching methods, and "blocking" the autofluorescent structures (Baschong et al. 2001; Billinton and Knight 2001). To date, none of these approaches has fully succeeded in part because effectiveness of the "blocking steps" varies by tissue type and method of processing (Baschong et al. 2001). For example, Viegas et al. (2007) concluded that a combination of short-duration, high-intensity ultraviolet (UV) irradiation (2 hr at 30 W) and Sudan Black B was the best protocol to reduce autofluorescence regardless of the extent of vascularity and level of lipofuscin content. A different approach was advocated by Niki et al. (2004), using a red fluorescence of PerCP on FFPE tissue sections, with specific red staining being clearly distinguishable from the yellow-green autofluorescent background. More recently, Robertson et al. (2008) reported a combined approach to multiple IF labeling of FFPE tissue, using AR pretreatment, an indirect IF staining method, and confocal laser scanning microscopy to circumvent autofluorescence.

AR Enhances Antigenicity and Reduces Autofluorescence for IF Staining

The use of AR as a procedure to enhance antigenicity for IHC in FFPE tissue has the additional advantage of reducing nonspecific autofluorescence (D'Ambra-Cabry et al. 1995). In this context, a "test battery" approach has been used to establish an optimal protocol for IF staining in FFPE tissue sections. For example, Long and Buggs (2008) tested three buffer solutions (100 mM Tris, pH 10; 0.05% citraconic anhydride; 10 mM citrate with 2 mM EDTA and 0.05% Tween 20, pH 6.2) for AR heating in a microwave oven and achieved satisfactory IF staining results for all three AR solutions. Bataille et al. (2006) successfully performed multiple IF staining in FFPE tissue sections by using 0.1 M sodium citrate buffer at pH 7.2, heating at 90C in a water bath for 40 min. Critical points for reducing background staining included selecting primary antibodies derived from a species other than that of the tissue tested, optimizing the concentration of antibodies carefully with incubation of slides at 4C overnight, and establishing optimal AR conditions.

The AR method has also been used to enhance the quality of multiple IF labeling or combined IF and fluorescence in situ hybridization (FISH) labeling in FFPE tissues. Ge et al. (2006) established an AR technique for combining IF and FISH labeling in FFPE sections using a microwave oven at a low power level of 4 (40%) for 3 cycles × 5 min, with a 1-min break between each cycle. The temperature reached 100C in the jar, and the method gave satisfactory IF and FISH staining signals with clean background. Xia et al. (2007) reported combining FISH and IF on monkey tissues, using a monkey Y chromosome–specific probe with IF staining of epithelial cell markers, with AR pretreatment at 96C in citrate buffer (pH 6.0). They recommended the use of AR as effective pretreatment to replace enzyme digestion.

The availability of affinity-purified antibodies to heat-denatured green fluorescent protein (GFP) may provide a useful approach for examination of tissues labeled by GFP. Nakamura et al. (2008) successfully performed IF labeling for GFP on heated formalin-fixed mouse brain tissue sections by using a novel polyclonal antibody to heat-denatured GFP. This method allows use of AR treatment in GFP-labeled tissue sections for multiplexed detection of IF signals.

Extraction of Nucleic Acids and Proteins from FFPE Tissues

Extraction of nucleic acids and proteins from FFPE tissues has been successfully accomplished in recent years, particularly after adaptation of heat-induced AR methods (Goelz et al. 1985; Dubeau et al. 1986; Shibata et al. 1988; Frank et al. 1996; Masuda et al. 1999; Coombs et al. 1999; Shi et al. 2002; Shi and Taylor 2010c; Fowler CB et al. 2010). The use of an AR approach was initiated based on evidence that formalin-induced modification of protein and of nucleic acid is similar (McGhee and von Hippel 1977a, 1977b; Masuda et al. 1999; Shi et al. 2001). Experimental evidence was forthcoming in support of this approach, describing remarkable improvement of signal for chromogenic in situ hybridization (CISH) and FISH (Oliver et al. 1997; Bull and Harnden 1999; Kitayama et al. 1999; Gu et al. 2000). In addition, other investigators have demonstrated the effectiveness of heating for extraction of nucleic acids from FFPE tissues. Our own group at the University of Southern California has conducted and published extensive studies of AR-based heating protocols for DNA/RNA extraction from FFPE tissues (Shi et al. 2002; Shi et al. 2004; Shi and Taylor 2010c).

DNA. After performing a serial study to test various chemicals at varying pH values in combination with heating, a simple protocol of boiling FFPE tissue sections in a solution of sodium hydroxide (NaOH) or potassium hydroxide (KOH) was identified (Shi et al. 2004). The effectiveness of a strong alkaline solution may depend on its ability to denature and hydrolyze proteins, breaking cell and nuclear membranes as well as disrupting cross-linkages caused by formalin fixation. The heat-induced retrieval protocol yields a better quality and quantity of DNA samples extracted from FFPE tissue sections than conventional methods of extraction, as tested by a real-time kinetic thermocycling (KTC)–PCR, using three primer pairs of the p53 gene ranging from 152-541 bp (Shi et al. 2002), and by an array-based comparative genomic hybridization (a-CGH; Shi and Taylor 2010c). In the latter study, DNA extracted from FFPE tissue sections by using a heat-induced retrieval protocol yielded equivalent or better

results than those obtained by using the conventional non-heating protocol, although DNA extracted from unfixed frozen tissue sections always showed better scores than DNA extracted from FFPE tissue sections. In conclusion, this AR-based extraction protocol may provide an alternative approach for DNA studies of FFPE tissue samples (Ferrari et al. 2007).

One persisting concern is that artifactual DNA sequence alterations have been reported after formalin fixation. Williams et al. (1999) reported finding up to one mutation artifact per 500 bases in FFPE tissue but noted that these artifacts are distinguishable from true mutations by confirmational sequencing of independent amplification products, in essence comparing the product of different batches. Quach et al. (2004) documented that damaged bases can be found in DNA extracted from FFPE tissues, but the products are still "readable" after in vitro translation synthesis by Taq DNA polymerase. They pointed out that appropriate caution should be exercised when analyzing small numbers of templates or cloned PCR products derived from FFPE tissue samples.

Future study: One potentially fruitful research direction is that further evaluation of the quality of DNA extracted from FFPE tissue suggests that improved AR-based extraction may eliminate these types of errors. Koshiba et al. (1993) studied the effect of different fixatives under variable conditions, using a model system of Lambda phage DNA and FFPE tissues, and found that tissue fixation by buffered formalin at 4C allowed extraction of high-quality DNA for Southern blot analysis. They also demonstrated that application of a modified tissue lysing buffer, containing 4 M urea, enabled extraction of high molecular weight DNA. S.-G. Fang et al. (2002) developed a technique using gradual dehydration and critical point drying for high-quality DNA extraction from old formalin-fixed tissue specimens. Their method allows successful extraction of DNA from animal tissues fixed in formalin for as long as 70 years, still yielding high-quality DNA (>194 kb). These pioneer studies provide encouragement for ongoing attempts on retrieval of nucleic acids that have been modified by formalin fixation.

RNA. RNA extracted from FFPE tissues is usually considered to be poor material for molecular analysis because of degradation and RNA fragmentation (Mikhitarian et al. 2004). However, recent work has demonstrated that RNA extracted from FFPE tissue may be amenable to RT-PCR (Rupp and Locker 1988; Jackson et al. 1990; Krafft et al. 1997; Liu H et al. 2002). Masuda et al. (1999) used low-temperature heating (70C) with routine commercial kits for extraction of RNA to examine formalin-induced modification of synthetic oligo RNA, as well as cellular RNA, and complementary DNA synthesized from RNA. They concluded that "the majority of RNA can be extracted from properly processed archival samples. Although chemical modification by formalin does not allow the direct application of extracted RNA to cDNA synthesis and RT-PCR, more than half of the modification is simple methylol addition, which is reversed by simply heating in TE buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA)." Subsequently, Hamatani et al. (2006) reported that preheating RNA extracted from long-term preserved FFPE tissues in citrate buffer (pH 4.0) resulted in significantly increased efficiency of RT-PCR. RNA extracted from archival FFPE tissues stored for as long as 21 years, with fragment sizes smaller than 60 bp, could be amplified by RT-PCR successfully at a rate of greater than 80%.

We recently conducted experiments to extract mRNA from a cell model (MBA-MB-486 cell line of human breast cancer) processed in both frozen and FFPE blocks, with fixation times ranging from 6 hr to 30 days (Shi and Taylor 2010c). Similar studies were extended to recently collected samples of human breast cancer tissues processed by OCT (Mile Laboratories, Elkhart, IN), embedding and snap-freezing and extraction of the corresponding routine FFPE block. For the frozen cell/tissue samples, RNA extraction was carried out by using the TRIzol reagent kit (Life Technologies, Grand Island, NY). For the paraffin-embedded cell/tissue, RNA extraction was carried out by two methods: heating and non-heating (using enzyme digestion and the TRIzol kit) for comparison. RT-PCR was performed to compare the results. A panel of primers designed to amplify mRNA sequences of 10 genes was used to examine the efficiency of the heat-induced RNA retrieval technique, essentially the same technique as used for DNA extraction from archival tissue sections (Shi et al. 2002). Briefly, following optimization studies, a total of 500 µl of Britton-Robinson (BR) buffer at pH 7.4 was added to each microtube containing a 20-µm FFPE tissue section and was heated at boiling condition for 20 min in lieu of conventional enzyme digestion. After heating treatment, the microtube was allowed to cool for 5 min at room temperature, prior to using the TRIzol reagent kit. The RNA extracted from FFPE tissue, by either the heating or the non-heating protocol, was sufficient in quantity and quality for successful application for RT-PCR tests to achieve amplicons up to a larger size of 461 bp in FFPE tissue fixed for up to 7 days.

Protein. Based on the principles of AR, a high-temperature heating protocol for protein extraction from FFPE tissue sections was initiated by Ikeda et al. (1998) and since has been widely adopted. Most, if not all, commercial reagents designed for protein extraction from FFPE tissue are derived from heat-induced AR techniques. One simple protocol of boiling FFPE tissue sections in a retrieval solution of Tris-HCl buffer (pH 7 or 9) containing 2% SDS was developed in our laboratory, and the quality of protein extracted was evaluated by mass spectrometry (MS; Shi et al. 2006). Subsequently, this protocol was successively adopted by other research groups (Fowler CB et al. 2007; Ono et al. 2009). C. B. Fowler et al. (2007) designed "tissue surrogates" simulating FFPE tissue to test efficiency of protein extraction

and found that the most effective protein extraction buffer was 20 mM Tris-HCl containing 2% SDS, outperforming several commercial products.

Heating Is the Most Important Factor for Protein Extraction from FFPE Tissue

In addition to ourselves, most investigators have emphasized high-temperature heating as the critical factor for protein extraction (Chu et al. 2005; Prieto et al. 2005; Yamashita and Okada 2005b; Fowler CB et al. 2010). Jiang et al. (2007) compared the efficiency of protein extraction from FFPE tissue between heating (at 100C for 30 min) and non-heating protocols and demonstrated that the protein concentration from the heated sample was much higher than that obtained by the non-heating sample (10.9 vs 0.82 mg/mL).

Currently, various chemicals are used in the "AR solution" for the heating process, such as SDS, dithiothreitol (DTT), guanidine, glycine, β-octylglucoside, and others, sometimes with additional procedures for homogenization of FFPE tissue (Fowler CB et al. 2007; Nirmalan et al. 2009; Addis et al. 2009; Azimzadeh et al. 2010). Hwang et al. (2007) demonstrated that SDS was a critical chemical for efficient protein extraction from FFPE tissue. To date, although extraction methods are similar, there is no consensus on a standard universal protocol for proteomic analysis in FFPE tissue.

Solubilization of FFPE Tissue Sections in the Process of Extraction: A Critical Point

Most investigators also agree that one key issue for improving the efficiency of protein extraction is the effectiveness of solubilization of FFPE tissue sections in the heating process (Murphy et al. 2001). Using a high-pressure apparatus under 45,000 psi high pressure, C. B. Fowler et al. (2008) demonstrated that an increase in hydrostatic pressure to augment heat treatment dramatically improved both the protein extraction efficiency (from 60–100%) and the reversal of formaldehyde-induced protein modifications (from 20–100%) in a model tissue system. In our experience, some mechanical procedures such as motor-driven pestle and/or sonication are helpful for homogenizing FFPE tissue sections, leading to a higher yield of proteins.

Further Study to Improve Quality and Quantity of Proteins for Proteomic Research

There is now general agreement that in any study of fixed tissues, protein extraction and preparation are the most critical steps for proteomics studies (Saravanan and Rose 2004; Fowler CB et al. 2010). Satisfactory extraction of proteins from FFPE tissue requires not only a sufficient quantity to meet all test needs but also high quality to represent the true protein expression profile of the cell/tissue examined. On the basis of a "tissue surrogate" model, C. B. Fowler et al. (2010) raised several critical questions pertaining to whether proteins extracted from FFPE tissue are sufficient or reliable for proteomics analysis. Mass spectrometry data comparing extracts from fresh tissue and FFPE tissue for the same cell/tissue sample (Table 2) support the notion that proteins extracted from FFPE tissue using heat-induced AR protocols are suitable for proteomics studies, as do data from protein array techniques (Prieto et al. 2005; Rahimi et al. 2006; Becker et al. 2007; Becker et al. 2009). For example, Balgley et al. (2009) tested 10 archival FFPE tissue blocks with variable storage periods, as long as 28 years, by "shotgun" proteomics and found that high confidence and comparative proteome analysis were achieved in even the oldest tissue. In this context, AR-based methods, borrowed from the IHC field, are in like manner extending the usefulness and value of archival materials stored as paraffin blocks.

Quantitative IHC Based on AR

Recent rapid development of prognostic markers and targeted cancer treatments based upon levels of expression on IHC findings, exemplified by HER2, have raised a new issue—namely, the need for true quantitative IHC (QIHC; Taylor 2006b; Taylor and Levenson 2006; Leong 2009; Moelans et al. 2010; Taylor et al. 2010). So called semi-quantitative methods exist but leave much to be desired in terms of reproducibility and consistency between laboratories and between pathologists who score the slides on a 0, +, ++, +++ scale. The issues of IHC standardization and the need for universal controls or quantifiable reference standards have emerged as critical issues that must be addressed in the development of QIHC techniques (Taylor 2001, 2006a, 2006b; Anagnostou et al. 2010; Shi et al. 2007; Shi, Roth, et al. 2010; Taylor and Levenson 2006; Taylor et al. 2010; Torlakovic et al. 2010).

Previous Studies Insisted on Identical Conditions of FFPE but Lacked a Quantifiable Standard

In the past two decades, all previous approaches dealing with QIHC were based on a principle that required an identical FFPE tissue-processing condition, resulting in the development of complicated but not practical methods and a lack of a quantifiable standard. During these years, numerous commercially available image analysis (IA) and autostainers have been developed and adopted, increasing the standardization of IHC by improved performance

Table 2. Quality Reliability of Proteins Extracted from FFPE Tissue[a]

Authors	Parameter	Results		Conclusion
		Fresh Tissue	FFPE Tissue	
Palmer-Toy et al. (2005)	Total confident proteins by MS	94	123	Proteins identified from FFPE tissue compare favorably to that obtained from fresh tissue.
Shi et al. (2006)	Distinct proteins by MS	2404	3263	Most proteins of two groups showed overlap. Plot distributions are in good agreement.
Guo et al. (2007)	Distinct proteins by MS	3110	2733	Eighty-three percent of FFPE proteins overlap with fresh ones.
Jiang et al. (2007)	Proteins identified with two peptides by MS	480	470	Proteins identified in two groups are very similar. Valuable data could be obtained from FFPE tissue by proteomics.
Becker et al. (2007)	HER2 analysis of 17 breast cancers by WB and RPPA, quantified in relative intensity[b]	Relative intensity = 0.70	Relative intensity = 0.71	No differences were found in protein yield and abundances between fresh and FFPE tissues.
Addis et al. (2009)	SDS-PAGE "pre-fractionation" cutting bands by MS[c]	85	66	Among top 50 proteins, 80% were identified in both tissues.
Sprung et al. (2009)	Total unique peptides identified by MS	12,265	10,349	Total protein groups identified in FFPE tissue were 90% of that in frozen tissue. It is feasible for proteomic analysis of FFPE tissue.

[a]Randomly selected articles published in recent years.
[b]Relative intensity was calculated as mean intensity value of HER2 signal from the second point of the dilution curve divided by the corresponding value from the Sypro Ruby–stained image.
[c]Authors did a careful comparative study using SDS-PAGE, Western blot (WB), enzyme-linked immunosorbent assay, reverse-phase protein array (RPPA), and mass spectrometry (MS) to validate the quality of proteins extracted from formalin-fixed paraffin-embedded (FFPE) tissue and achieved satisfactory results for all tests.

of IHC protocols and more consistent interpretation of semi-quantitative findings (Floyd 2010; Hicks and McMahon 2010). For more than 10 years, experienced pathologists have advocated the use of image analysis for QIHC based on the advantages of consistent evaluation of IHC intensity or more precise counting of the positively stained cells (Kaczmarek et al. 2004; Kaczmarek et al. 2008). However, although IA does provide accurate measurement of intensity and accurate scoring of cell numbers, the method still is unable to measure exact amounts of protein targets in cells or tissues because of the lack of a "standard ruler" (calibration control) that can convert the intensity of the IHC signal into measurement of protein by weight. This is the key point of QIHC. Currently, IA-generated quantitation of IHC is based on optical density or counting the amount of chromogens per pixel, but actual amounts of protein by weight cannot be calculated (Aziz 1992; Grandis et al. 1996; Matkowskyj et al. 2003). Press et al. (1993) published a method of QIHC for detecting HER2 in FFPE tissue sections based on Western blotting. A similar principle is embodied in the "Quicgel" method (Riera et al. 1999). It created an internal artificial tissue control block using a breast cancer cell line that is added to the tissue cassette containing the clinical biopsy specimen. The Quicgel method, however, has proven not to be practical for routine use because of logistical issues and of course is not applicable for retrospective studies on archival tissue. Allred et al. (2006) also proposed a QIHC method based on a variation of the enzyme-linked immunosorbent assays (ELISA) technique, in which the FFPE section was first stained by IHC, and the chromogen was then leached out for measurement by an ELISA-like method. However, in this method, the unknown amount of protein loss in sample preparation remains a problem. A protein-embedding approach has been proposed by us (Shi et al. 2005) based on the previous documented use of purified protein incorporated within various matrices as a model system for cytochemistry (van der Ploeg and Duijndam 1986). Although FFPE protein-coated beads have been developed to obtain preliminary results, issues remain pertaining to the optimal material for use as a matrix, and FFPE processing of protein-coated beads is still not identical to that of the protein of interest in FFPE sections that are used for diagnosis. Currently, there is no practical method that can be used for QIHC in archival FFPE tissue sections. Theoretical considerations aside, the immediacy of a current need for standardization of IHC requires some practical short-term action, even as work proceeds to a scientifically based solution. In this context, the recent guidelines published from the American Society of Clinical

Oncology/College of American Pathologists (ASCO/CAP; Wolff et al. 2007) attempt to introduce more uniformity in sample preparation for specific IHC tests, specifically IHC for HER2. The work of UKNEQAS (United Kingdom National External Quality Assessment Scheme for IHC) has demonstrated that such guidelines, if effectively monitored for compliance, do produce improvements in reproducibility of IHC findings (Rhodes et al. 2000; Rhodes et al. 2002) but continue to fall short in the sense that sample preparation can realistically never be the same for all tissues in all laboratories.

AR-IHC Staining Intensity: FFPE Is Comparable to Frozen Tissue Section

From a practical point of view, one of the most difficult issues in the standardization of IHC on FFPE tissues is the adverse and variable influence of formalin fixation on IHC detection, resulting in a major uncontrollable intrinsic factor. Based on our and other investigators' studies, one proposal is to minimize variation of IHC staining signals among hundreds and thousands of FFPE tissue sections by using optimal AR protocols (Shi et al. 2007). Briefly, the hypothesis is that use by each laboratory of individually optimized AR protocols provides a potential approach to reach a comparable (if not identical) level of IHC staining, following variable conditions of FFPE sample preparation (Shi et al. 1998; Shi et al. 1999; Shi, Gu, Cote, et al. 2000; Shi et al. 2007; Boenisch 2005). We recognize that this is a pragmatic, empirical approach that ignores the finer points of the diverse effects of fixation and processing on different proteins. However, it has the advantage of being relatively simple and inexpensive and is perhaps the most practical and immediate approach to improve reproducibility of IHC for archival FFPE tissue sections. Several articles published in recent years have demonstrated feasibility on a semi-quantitative basis (Rhodes et al. 2000; Rhodes et al. 2002; Jacobs et al. 2000; Boenisch 2005; Moon et al. 2008), although further studies are required to reach the goal of true QIHC, defined as the measurement of analytes (proteins) by weight.

Evidence suggests that many proteins (antigens) "retrieved" from FFPE tissue sections show substantial, if not complete, restoration of immunoreactivity, expressed as an AR efficiency that may approach 100%, exemplified by ER, progesterone receptor (PR), HER-2/neu, p53, and Ki-67 (MIB-1). For a number of proteins, the literature demonstrates high concordance between IHC findings and biochemical measurement in the same specimen (MacGrogan et al. 1996; Pertschuk and Axiotis 2000). In addition, many investigators have demonstrated comparable IHC staining results between frozen and FFPE tissue sections following AR (Hawes et al. 2010; Taylor et al. 2010), to the extent that there is a case for arguing that AR-IHC may serve as the "gold standard" (Shi et al. 2008; Shidham et al. 2000). However, there is a lack of rigorous data that demonstrate the extent of "loss" of immunoreactivity of specific proteins during fixation and sample preparation. Similarly, the exact degree to which the protein is restored by the retrieval process remains unknown. Are any proteins "retrieved" with 100% efficiency? If so, which ones, and what is the efficiency of retrieval of key proteins that are used as prognostic and predictive markers? Answering these questions requires strict quantification—namely, measurement by weight—of individual proteins in defined tissues when fresh and during various phases of fixation and retrieval. Some semi-quantitative work has been done to support feasibility of this task, but accurate objective data do not yet exist.

Perplexingly, the ability to successfully recover protein antigens using AR methods optimized by using the "test battery" approach has gone relatively unnoticed. However, IHC of FFPE tissue sections subjected to optimal AR treatment, in most conditions, yields an IHC intensity in FFPE tissue sections that is identical to that obtained from the corresponding frozen tissue section (Arber 2002; Boenisch 2005; Shi et al. 2007; Shi et al. 2008). We compared IHC staining results between frozen and FFPE tissue sections using 26 commonly used primary antibodies and found that all 26 antibodies showed the strongest IHC intensity identical to the IHC intensity of frozen tissue sections (Figure 1; Shi et al. 2008). In recent years, the FFPE tissue section with use of an optimal AR treatment has quietly been accepted as the gold standard for many IHC studies. For example, Shidham et al. (2003) 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 sample preparation. They used FFPE tissue sections with AR treatment as the standard positive control to select the optimal method of sample preparation and demonstrated that FFPE tissue sections with use of AR yielded the best IHC staining results.

The opportunity of scientific discovery is always surrounding us. One misunderstanding goes unnoticed because of an accepted customary concept. More than 20 years ago, a concept that heating cell/tissue samples over 65C denatures proteins kept people from thinking that heat treatment on FFPE tissue section could be used as a simple retrieval method (Shi and Taylor 2010d). In fact, the effect of formalin-induced protein modification protects proteins from being denatured during heating. Today, a similar perception has set in and thus has created a similar dilemma. The idea that a protein cannot be used as a "benchmark" unless it has been processed and treated exactly as an FFPE tissue has hindered the creation of a simple method of QIHC. The dramatic change that can occur by AR-IHC- induced identical staining intensity between FFPE and frozen tissue has gone unnoticed. Based on our method of optimized AR, it should be possible to develop a simple method for QIHC by using

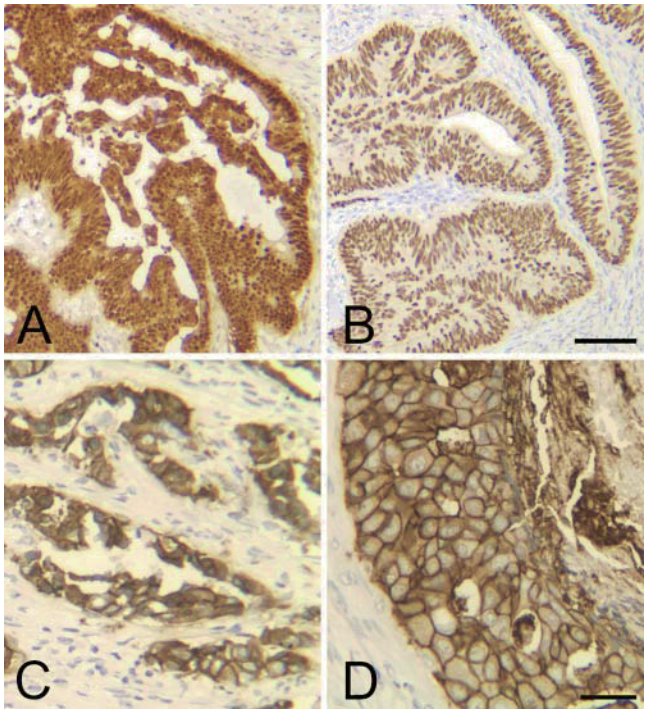

Figure 1. Comparison of immunohistochemistry staining intensity of (A, B) p53 and (C, D) HER2 between (A, C) frozen and (B, D) formalin-fixed paraffin-embedded tissue sections showing comparable intensity. (A, B) Colon cancer tissue. (C, D) Breast cancer tissue. Bars: A, B = 50 µm; C, D = 25 µm.

a standard protein control instead of the previous complicated methods described above.

From ELISA to QIHC: A Simple and Inexpensive Approach

Compared to ELISA, the IHC method is based on the same principle of antigen/antibody recognition but for a different form of the antigen due to formalin fixation and histological processing. ELISA has long been used as a reliable quantitative method to measure the amount of protein in samples based on comparison with a standard calibration control, which provides a standard reference curve ("standard ruler"). A major reason why IHC cannot be used to measure the exact amount of protein in a cell/tissue section is the lack of a comparable standard ruler. As described above, a dramatic change of AR-IHC staining result has been ignored by most investigators who would rather develop complicated methods based on the principle of an identical condition of FFPE process to match that of tested FFPE tissue samples.

Hypothesis. Our hypothesis was documented previously (Shi et al. 2007): The use of optimized AR protocols will permit a defined and reproducible IHC intensity for a specific protein in FFPE tissues (the percentage of retrieved IHC intensity by AR, expressed as AR "efficiency," named as the AR rate or *R*%), and it can be determined with reference to the IHC intensity generated for the same protein present in the original fresh/unfixed tissue. This hypothesis may also be presented mathematically: The protein amount in a fresh cell/tissue, designated as *Pf*, produces a total IHC signal in fresh tissue of $\int (Pf)$. When the identical IHC staining protocol plus AR treatment is applied to an FFPE section of the same tissue, the IHC signal is $\int (Pffpe)$. The AR rate (*R*%) is calculated as $R\% = \int (Pffpe) / \int (Pf) \times 100\%$. The amount of protein in the FFPE tissue may then be derived as follows: If AR is perfectly efficient, then $R\% = 100\%$ and $Pffpe = Pf$; if AR recovers less than 100% of the given protein, then $R\% < 100\%$. The value of *R*% (as determined by experimental observation for each specific protein) is used to compensate for the reduced IHC intensity in FFPE to calculate the exact amount of protein existing in the identical fresh cell/tissue sample.

Rationale. Through designed cell/tissue models the goal is to test the hypothesis described above and to establish a practical and inexpensive method of QIHC for archival FFPE tissues based on the principles of the ELISA method. If ELISA can be used to convert the immunoreactivity (optimal density [OD] readout) into the amount of tested protein by a standard curve, then IHC can also convert the IHC intensity of FFPE tissue sections into the amount of tested protein by an equivalent standard ruler. The IHC intensity of FFPE tissue sections can be recovered to a level that is identical to that obtained by frozen tissue sections by using an optimized AR protocol (Arber 2002; Boenisch 2005; Shi et al. 2007). Therefore, it is reasonable to propose that the amount of a protein (antigen) existing in the FFPE tissue section can be measured by an appropriate serially diluted protein slide used as a standard ruler and by using the AR rate (*R*%) for compensation if $R\% < 100\%$ or by direct comparison if $R\% = 100\%$. Because this is a novel QIHC method, a careful step-by-step experiment must be performed to demonstrate the following: (1) The standard ruler can be used satisfactorily with fresh cell/tissue models; (2) FFPE and fresh cell/tissue models must be strongly correlated based on both IHC and protein extraction, allowing for the determination of the AR rate (*R*%) as described above; and (3) there is final validation of a strong correlation between the standard ruler and FFPE tissue sections based on cell/tissue model and further demonstration by clinical cases. To do so, it is critical to focus on two major issues: protein extraction and accurate measurement of proteins extracted from cell/model systems. A finalized QIHC method is based on two positive controls: (1) an FFPE cell/tissue section under identical conditions as matched with test tissue sections (routine control) and (2) a quantitative standard consisting of a known amount of serially diluted protein used as a standard ruler to measure the amount of protein in FFPE tissue sections. Thus, when the first control indicates that an optimal AR-IHC procedure has been performed, the amount of the target protein can be read out from the standard

ruler provided by the second control. IA software will be adopted to quantitate the IHC staining result by using the standard ruler following the same method of ELISA.

Mechanism of AR

In many ways, it is remarkable that AR has achieved such wide acceptance, lacking any comprehensive understanding of its mechanism of action. But this fact becomes less surprising in the context of a similar or even greater lack of understanding of the exact mechanisms of formalin fixation itself. It appears logical, if belated, that further study of the mechanisms of fixation and retrieval may lead to scientifically well-grounded recommendations for standardization.

With respect to the AR technique, several hypotheses pertaining to the mechanism have been proposed (Shi et al. 1997; Shi, Gu, Turrens, et al. 2000), and recent studies have shed some light (Rait, O'Leary, et al. 2004; Rait, Xu, et al. 2004; Sompuram AR et al. 2004; Sompuram SR et al. 2006; Yamashita 2007). The research group of AFIP (Armed Forces Institute of Pathology), led by Mason, O'Leary, and others, has taken a unique approach to understanding the mechanisms of heat-induced AR. As we know, the immunoreactivity of protein is related to its chemical and conformational properties. In their work, they used bovine pancreatic ribonuclease A (RNase A) as a model (Rait, O'Leary, et al. 2004; O'Leary et al. 2009; Mason et al. 2010) and demonstrated the restoration of RNase A immunoreactivity by heating correlates, with the reversal of the chemical effects of formalin on the RNase A sample. Thus, reversal of protein formaldehyde adducts and cross-links are almost certainly fundamental to the success of the AR technique. Bogen, Sompuram, and colleagues conducted similar fundamental work using synthesized peptides as a model system for simulating the loss of immunoreactivity after fixation (Sompuram SR et al. 2006; Bogen et al. 2009; Bogen and Sompuram 2010). They discovered that most peptide epitopes in isolation did not lose immunoreactivity after treatment with formalin but did lose immunoreactivity if an irrelevant protein was added, possibly blocking the peptide epitope by formaldehyde-induced protein cross-linking. Heat-induced AR restored immunoreactivity. In their study, the precise amino acid composition of an epitope appears to be an important determinant of the precise conditions that are required for successful AR. Yamashita and others (Yamashita and Okada 2005b; Yamashita 2010) confirmed previous data that the pH and ionic strength of the retrieval solution are critical factors for AR efficiency in a mouse model, comparing SDS-PAGE gels of proteins and AR solutions at pH values ranging from 3 to 10.5.

In general, most investigators believe that breaking formalin-induced cross-linkages by heating may be the basic mechanism of AR. On the other hand, Kakimoto et al. (2008) proposed an additional mechanism for AR based on a study of IHC staining on fresh unfixed rat uterus tissue sections and dot-blot assay of rat protein extracts without formalin fixation. They observed that nine antibodies showed negative IHC staining results in unfixed tissues but were clearly positive after AR treatment. In addition, dot-blot assay was greatly enhanced by heating the protein-blotted membrane. They hypothesized that antibody accessibility to the target epitopes is limited by natural steric barriers caused by the antigenic protein itself, even in the fresh state. Clearly, this is an area for future research.

Future study. O'Leary et al. (2010) also suggested a set of directions for future research in AR techniques, focusing on two points: (1) the recovery of unmodified proteins in native conformation from FFPE tissue and (2) the development of new techniques for assessing the quantity and functional state of tissue proteins recovered from FFPE tissue. These recommendations reinforce the opinion of the authors that further development of the AR technique must based on a better scientific understanding of the molecular mechanisms and that this represents the key pathway to improved cell/tissue sample preparation, standardization of AR-IHC, and superior methods of extraction of nucleic acids/proteins for tissue proteomics. After 20 years, the AR technique is in many ways still in the developing stage. There is a pressing need for hard quantitative data, and the techniques are becoming available to move in this direction.

Tissue Proteomics: Symbiosis of AR-IHC and MS

Tissue-based proteomics offers a potential method for analyzing proteins in FFPE tissues independent of IHC methods. Comparing the findings of the two methods is likely to be productive in validation, each of the other, and in developing a better understanding of the issues of identifying proteins after FFPE processing. Not surprisingly, considering that both methods share a common start point—namely, FFPE tissues—both methods have resorted to AR-based techniques for preparing protein for analysis, either by extraction or in situ by IHC. As noted previously, many articles (Table 2) have demonstrated the availability of proteins extracted from FFPE tissue by methods using the heat-induced AR principle. The effectiveness of this approach has provided potent stimulus to the field of extraction proteomics, somewhat analogous to the rapid expansion of IHC following the introduction of AR for archival FFPE tissue 20 years ago. Further impetus is derived from the growing use of biomarkers as a facet of targeted therapy and "personalized" medicine (Kroll et al. 2008; Becker et al. 2009; Fang X et al. 2009; Gnanapragasam 2010; Berg et al. 2010). MS-based proteomics offers avenues for discovery by virtue of massive multiplexing capability, facilitating the detection of many thousands of proteins from a single routine FFPE tissue section. Reverse-phase protein arrays (RPPAs) offer different capabilities in the detection of substantially intact proteins in extracts of FFPE tissues (Berg et al. 2010). These types of studies yield

protein profiles that may serve to guide pathologists in the selection of targets that are expected to be demonstrable by IHC in FFPE sections of the same or similar tissues. Discovery may be conducted in a prospective or retrospective manner. For example, prospective comparisons may be performed of morphologically defined disease types in an effort to define potential molecular markers to be used for diagnostic purposes. Also, retrospective comparisons may be performed of biopsies collected from clinical trials in an attempt to define differences in protein status or expression that may correlate with therapeutic response.

For pathologists and morphologists, "seeing is believing," and most cancers still are diagnosed by morphologic methods. For these reasons, microscopy remains a very valuable tool to demonstrate cellular and subcellular localization of proteins for research and for diagnosis. IHC methods have proven difficult to quantify, whereas extraction proteomics may yield precise measurement of proteins by weight, down to the attogram level (Becker and Taylor 2010). Combining the two methods may yield a more objective approach for diagnostic pathology. IHC has the unique advantage of sharply localizing specific proteins in an exact cell/tissue component. Extraction proteomics can measure the precise amount of protein present in a cell population defined by morphology. IHC has the capacity to reveal the precise morphological distribution of several proteins in a critical signaling pathway in situ, and extraction proteomics can measure accurately the relative amounts present in different clinical situations. The methods combined thereby provide a scientific demonstration of expression of interacting proteins in normal and abnormal tissue structures, accurately quantified and precisely localized (Taylor et al. 2010; Becker and Taylor 2010).

AR–Imaging Mass Spectrometry: An Exciting Application of AR for FFPE Tissue Section

In most recent applications of AR for imaging mass spectrometry (IMS), exciting achievements have provided an avenue to fully analyze the proteome of archival FFPE tissue. Use of AR protocol for IMS is critical to generate excellent peptide IMS data with high resolution that opens a door to IMS analysis for using FFPE tissue sections (Ronci et al. 2008; Groseclose et al. 2008; Gustafsson et al. 2010).

There is general agreement that final validation of a biomarker requires IHC-assisted demonstration (Balgley 2010; Xu et al. 2008). Further studies combining proteomics by RPPA or MS, including IMS (Mukai and Setou 2010) and IHC, are likely to lead to the refinement of both methods in the analysis of FFPE tissues. The end result may be the creation of a broader field that defines and quantifies protein expression at a cellular level, incorporating the advantages of the wide spectrum of proteins demonstrable by MS or RPPA and the precise localization offered by IHC that may lead to a new era in terms of tissue proteomics (Shi, Balgley, et al. 2010).

Acknowledgement

We greatly appreciated Ms. Leslie K. Garcia, HT, our technician's excellent work of protein extraction and other laboratory work to support this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research and/or authorship of this article: Supported by NIH grant 1 R42 CA122715 from the Innovative Molecular Analysis Technologies (IMAT) Program.

References

Abbondanzo SL, Allred DC, Lampkin S, Banks PM. 1990. Enhancement of immunoreactivity among lymphoid malignant neoplasms in paraffin embedded tissues by refixation in zinc sulfate-formalin. Arch Pathol Lab Med. 115:31-33.

Abendroth CS, Dabbs DJ. 1995. Immunocytochemical staining of unstained versus previously stained cytologic preparations. Acta Cytol. 39:379-386.

Adams JC. 1992. Biotin amplification of biotine and horseradish peroxidase signals in histochemical stains. J Histochem Cytochem. 40:1457-1463.

Addis MF, Tanca A, Pagnozzi D, Crobu S, Fanciulli G, Cossu-Rocca P, Uzzau S. 2009. Generation of high-quality protein extracts from formalin-fixed, paraffin-embedded tissues. Proteomics. 9:3815-3823.

Allred DC, Mohsin SK, Genty C, Mao S. 2006. Method of quantitative immunohistochemistry and in situ hybridization. United States patent US 2006/0160151. July 20, 2006.

Anagnostou VK, Welsh AW, Giltnane JM, Siddiqui S, Liceaga C, Gustavson M, Syrigos KN, Reiter JL, Rimm DL. 2010. Analytic variability in immunohistochemistry biomarker studies. Cancer Epidemiol Biomarkers Prev. 19:982-991.

Arber DA. 2002. Effect of prolonged formalin fixation on the immunohistochemical reactivity of breast markers. Appl Immunohistochem Mol Morphol. 10:183-186.

Azimzadeh O, Barjaktarovic Z, Michaela A, Calzada-Wack J, Sarioglu H, Atkinson M, Tapio S. 2010. Formalin-fixed paraffin-embedded (FFPE) proteome analysis using gel-free and gel-based proteomics. J Proteome Res. 9:4710-4720.

Aziz DC. 1992. Quantitation of estrogen and progesterone receptors by immunocytochemical and image analyses. Am J Clin Pathol. 98:105-111.

Balgley BM. 2010. Application of shotgun proteomics to formalin-fixed and paraffin-embedded tissues. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 347-367.

Balgley BM, Guo T, Zhao K, Fang X, Tavassoli FA, Lee CS. 2009. Evaluation of archival time on shotgun proteomics of formalin-fixed and paraffin-embedded tissues. J Proteome Res. 8:917-925.

Baschong W, Suetterlin R, Laeng RH. 2001. Control of autofluorescence of archival formaldehyde-fixed, paraffin-embedded tissue in confocal laser scanning microscopy (CLSM). J Histochem Cytochem. 49:1565-1571.

Bataille F, Troppmann S, Klebl F, Rogler G, Stoelcker B, Hofstadter F, Bosserhoff A-K, Rummele P. 2006. Multiparameter immunofluorescence on paraffin-embedded tissue sections. Appl Immunohistochem Mol Morphol. 14:225-228.

Becker K-F, Schott C, Hipp S, Metzger V, Porschewski P, Beck R, Nährig J, Becker I, Höfler H. 2007. Quantitative protein analysis from formalin-fixed tissues: implications for translational clinical research and nanoscale molecular diagnosis. J Pathol. 211:370-378.

Becker K-F, Taylor CR. 2010. 'Liquid morphology': immunochemical analysis of proteins extracted from formalin fixed paraffin embedded tissues: combining proteomics with immunohistochemistry. Appl Immunohistochem Mol Morphol. Sep 7 [Epub ahead of print]

Becker K-F, Walch A, Ueffing M. 2009. Protein analysis of tissues: current views and clinical perspectives. Virchows Arch. 455:191-192.

Berg D, Hipp S, Malinowsky K, Böllner C, Becker KF. 2010. Molecular profiling of signalling pathways in formalin-fixed and paraffin-embedded cancer tissues. Eur J Cancer. 46:47-55.

Billinton N, Knight AW. 2001. Seeing the wood through the trees: a review of techniques for distinguishing green fluorescent protein from endogenous autofluorescence. Anal Biochem. 291:175-197.

Boenisch T. 2005. Effect of heat-induced antigen retrieval following inconsistent formalin fixation. Appl Immunohistochem Mol Morphol. 13:283-286.

Bogen SA, Sompuram SR. 2010. A linear epitopes model of antigen retrieval. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 287-302.

Bogen SA, Vani K, Sompuram SR. 2009. Molecular mechanisms of antigen retrieval: antigen retrieval reverses steric interference caused by formalin-induced cross-links. Biotech Histochem. 84:207-215.

Boon ME, Beck S, Kok LP. 1995. Semiautomatic PAPNET analysis of proliferating (MiB-1-positive) cells in cervical cytology and histology. Diagn Cytopathol. 13:423-428.

Boon ME, Kleinschmidt-Guy ED, Ouwerkerk-Noordam E. 1994. PAPNET for analysis of proliferating (MIB-1 positive) cell populations in cervical smears. Eur J Morphol. 32:78-85.

Boon ME, Kok LP. 1995. Breakthrough in pathology due to antigen retrieval. Mal J Med Lab Sci. 12:1-9.

Boon ME, Kok LP, Suurmeijer AJH. 2000. The MIB-1 method for fine-tuning diagnoses in cervical cytology. In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 57-70.

Brown G, Tao L. 1992. Restoration of broken cytology slides and creation of multiple slides from a single smear preparation. Acta Cytol. 36:259-268.

Bull JH, Harnden P. 1999. Efficient nuclear FISH on paraffin-embedded tissue sections using microwave pretreatment. BioTechniques. 26:416-422.

Chivukula M, Dabbs DJ. 2010. Immunocytology. In: Dabbs DJ, editor. Diagnostic immunohistochemistry: theranostic and genomic applications. 3rd ed. Philadelphia: Saunders-Elsevier.

Chu W-S, Liang Q, Liu J, Wei MQ, Winters M, Liotta L, Sandberg G, Gong M. 2005. A nondestructive molecule extraction method allowing morphological and molecular analyses using a single tissue section. Lab Invest. 85:1416-1428.

Colvin RB, Bhan AK, McCluskey RT. 1995. Diagnostic immunopathology. New York: Raven.

Coombs NJ, Gough AC, Primrose JN. 1999. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. Nucleic Acids Res. 27:e12-e17.

Dabbs DJ. 2008. Immunohistochemical protocols: back to the future. Am J Clin Pathol. 129:355-356.

Dabbs DJ, editor. 2010. Diagnostic immunohistochemistry: theranostic and genomic applications. 3rd ed. Philadelphia: Saunders-Elsevier.

Dabbs D, Wang X. 1998. Immunocytochemistry on cytologic specimens of limited quantity. Diagn Cytopathol. 18:166-169.

D'Ambra-Cabry K, Deng DH, Flynn KL, Magee KL, Deng JS. 1995. Antigen retrieval in immunofluorescent testing of bullous pemphigoid [see comments]. Am J Dermatopathol. 17: 560-563.

D'Amico F, Skarmoutsou E, Stivala F. 2009. State of the art in antigen retrieval for immunohistochemistry. J Immunol Methods. 341:1-18.

DeLellis R, Hoda R. 2006. Immunochemistry and molecular biology in cytological diagnosis. In: Koss L, Melamed M, editors. Koss' diagnostic cytology and its histopathologic bases. Philadelphia: Lippincott Williams & Wilkins. p. 1635-1680.

Dubeau L, Chandler LA, Gralow JR, Nichols PW, Jones PA. 1986. Southern blot analysis of DNA extracted from formalin-fixed pathology specimens. Cancer Res. 46:2964-2969.

Eltoum I, Fredenburgh J, Myers RB, Grizzle WE. 2001. Introduction to the theory and practice of fixation of tissues. J Histotechnol. 24:173-190.

Evers P, Uylings HB. 1994. Effects of microwave pretreatment on immunocytochemical staining of vibratome sections and tissue blocks of human cerebral cortex stored in formaldehyde fixative for long periods. J Neurosci Methods. 55:163-172.

Evers P, Uylings HBM. 2000. Microwave-stimulated antigen retrieval in neuroscience. In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 139-150.

Fang S-G, Wan Q-H, Fujihara N. 2002. Formalin removal from archival tissue by critical point drying. BioTechniques. 33:604-611.

Fang X, Balgley BM, Lee CS. 2009. Recent advances in capillary electrophoresis-based proteomic techniques for biomarker discovery. Electrophoresis. 30:3998-4007.

Ferrari HF, Luvizottob MCR, Rahald P, Cardoso TC. 2007. Detection of bovine Herpesvirus type 5 in formalin-fixed, paraffin-embedded bovine brain by PCR: a useful adjunct to conventional tissue-based diagnostic test of bovine encephalitis. J Virol Methods. 146:335-340.

Fetsch PA, Abati A. 2001. Immunocytochemistry in effusion cytology. Cancer. 93:293-308.

Fetsch PA, Abati A. 2007. The effects of antibody clone and pretreatment method on the results of HER2 immunostaining in cytologic samples of metastatic breast cancer: a query and a review of the literature. Diagn Cytopathol. 35:319-328.

Fetsch PA, Simsir A, Brosky k, Abati A. 2002. Comparison of three commonly used cytologic preparations in effusion immunocytochemistry. Diagn Cytopathol. 26:61-66.

Floyd AD. 2010. Image analysis in immunohistochemistry. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 165-185.

Fowler CB, Cunningham RE, O'Leary TJ, Mason JT. 2007. 'Tissue surrogates' as a model for archival formalin-fixed paraffin-embedded tissues. Lab Invest. 87:836-846.

Fowler CB, Cunningham RE, Waybright TJ, Blonder J, Veenstra TD, O'Leary TJ, Mason JT. 2008. Elevated hydrostatic pressure promotes protein recovery from formalin-fixed, paraffin-embedded tissue surrogates. Lab Invest. 88:185-195.

Fowler CB, O'Leary TJ, Mason JT. 2010. Techniques of protein extraction from FFPE tissue/cells for mass spectrometry. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 335-346.

Fowler LJ, Lachar WA. 2008. Application of immunohistochemistry to cytology. Arch Pathol Lab Med. 132:373-383.

Fraenkel-Conrat H, Brandon BA, Olcott HS. 1947. The reaction of formaldehyde with proteins: IV. Participation of indole groups. J Biol Chem. 168:99-118.

Fraenkel-Conrat H, Olcott HS. 1948a. The reaction of formaldehyde with proteins: V. Cross-linking between amino and primary amide or guanidyl groups. J Am Chem Soc. 70:2673-2684.

Fraenkel-Conrat H, Olcott HS. 1948b. Reaction of formaldehyde with proteins: VI. Cross-linking of amino groups with phenol, imidazole, or indole groups. J Biol Chem. 174:827-843.

Frank TS, Svoboda-Newman SM, Hsi ED. 1996. Comparison of methods for extracting DNA from formalin-fixed paraffin sections for nonisotopic PCR. Diagn Mol Pathol. 5:220-224.

Fulciniti F, Frangella C, Staiano M, La Vecchia F, Botti G, Demuru A, Magliulo G, Boon ME. 2008. Air-dried smears for optimal diagnostic immunocytochemistry. Acta Cytol. 52:178-186.

Furuya T, Ikemoto K, Kawauchi S, Oga A, Tsunoda S, Hirano T, Sasaki K. 2004. A novel technology allowing immunohistochemical staining of a tissue section with 50 different antibodies in a single experiment. J Histochem Cytochem. 52:205-210.

Ge S, Crooks GM, McNamara G, Wang X. 2006. Fluorescent immunohistochemistry and in situ hybridization analysis of mouse pancreas using low-power antigen-retrieval technique. J Histochem Cytochem. 54:843-847.

Gnanapragasam VJ. 2010. Unlocking the molecular archive: the emerging use of formalin-fixed paraffin-embedded tissue for biomarker research in urological cancer. BJU Int. 105:274-278.

Goel MM, Mehrotra A, Singh U, Gupta HP, Misra JS. 2005. MIB-1 and PCNA immunostaining as a diagnostic adjunct to cervical Pap smear. Diagn Cytopathol. 33:15-19.

Goelz SE, Hamilton SR, Vogelstein B. 1985. Purification of DNA from formaldehyde-fixed and paraffin-embedded tissue. Biochem Biophys Res Commun. 130:118-126.

Gong Y, Joseph T, Sneige N. 2005. Validation of commonly used immunostains on cell-transferred cytologic specimens. Cancer. 105:158-164.

Gong Y, Symmans WF, Krishnamurthy S, Patel S, Sneige N. 2004. Optimal fixation conditions for immunocytochemical analysis of estrogen receptor in cytologic specimens of breast carcinoma. Cancer. 102:34-40.

Gown AM. 2004. Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. Am J Clin Pathol. 121:172-174.

Gown AM, de Wever N, Battifora H. 1993. Microwave-based antigenic unmasking: a revolutionary new technique for routine immunohistochemistry. Appl Immunohistochem. 1:256-266.

Grandis JR, Melhem MF, Barnes EL, Tweardy DJ. 1996. Quantitative immunohistochemical analysis of transforming growth factor- and epidermal growth factor receptor in patients with squamous cell carcinoma of the head and neck. Cancer. 78:1284-1292.

Groseclose MR, Massion PP, Chaurand P, Caprioli RM. 2008. High-throughput proteomic analysis of formalin-fixed paraffin-embedded tissue microarrays using MALDI imaging mass spectrometry. Proteomics. 8:3715-3724.

Gu J, Farley R, Shi S-R, Taylor CR. 2000. Target retrieval for in situ hybridization. In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 115-128.

Guo T, Wang W, Rudnick PA, Song T, Li J, Zhuang Z, Weil RJ, DeVoe DL, Lee CS, Balgley BM. 2007. Proteome analysis of microdissected formalin-fixed and paraffin-embedded tissue specimens. J Histochem Cytochem. 55:763-772.

Gustafsson JOR, Oehler MK, McColl SR, Hoffmann P. 2010. Citric acid antigen retrieval (CAAR) for tryptic peptide imaging directly on archived formalin-fixed paraffin-embedded tissue. J Proteome Res. 9:4315-4328.

Hamatani K, Eguchi H, Takahashi K, Koyama K, Mukai M, Ito R, Taga M, Yasui W, Nakachi K. 2006. Improved RT-PCR amplification for molecular analyses with long-term preserved formalin-fixed, paraffin-embedded tissue specimens. J Histochem Cytochem. 54:773-780.

Hausen P, Dreyer C. 1982. Urea reactivates antigens in paraffin sections for immunofluorescent staining. Stain Technol. 57:321-324.

Hawes D, Shi S-R, Dabbs DJ, Taylor CR, Cote RJ. 2010. Immunohistochemistry. In: Weidner N, Cote RJ, Suster S, Weiss LM, editors. Modern surgical pathology. 2nd ed. New York: Elsevier. p. 48-70.

Hicks DG, McMahon L. 2010. The pros and cons of automation for immunohistochemistry from the prospective of the pathology laboratory. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 151-163.

Hsu S-M, Raine L, Fanger H. 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem. 29:577-580.

Huang S-N. 1975. Immunohistochemical demonstration of hepatitis B core and surface antigens in paraffin sections. Lab Invest. 33:88-95.

Hwang S-I, Thumar J, Lundgren DH, Rezaul K, Mayya V, Wu L, Eng J, Wright ME, Han DK. 2007. Direct cancer tissue proteomics: a method to identify candidate cancer biomarkers from formalin-fixed paraffin-embedded archival tissues. Oncogene. 26:65-76.

Ikeda K, Monden T, Kanoh T, Tsujie M, Izawa H, Haba A, Ohnishi T, Sekimoto M, Tomita N, Shioaki H, et al. 1998. Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. J Histochem Cytochem. 46:397-404.

Ino H. 2003. Antigen retrieval by heating En Bloc for pre-fixed frozen material. J Histochem Cytochem. 51:995-1003.

Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P. 1990. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. J Clin Pathol. 43:499-504.

Jacobs TW, Gown AM, Yaziji H, Barnes MJ, Schnitt SJ. 2000. HER-2/neu protein expression in breast cancer evaluated by immunohistochemistry: a study of interlaboratory agreement. Am J Clin Pathol. 113:251-258.

Jagirdar J. 2008. Immunohistochemistry, then and now. Arch Pathol Lab Med. 132:323-325.

Jiang X, Jiang X, Feng S, Tian R, Ye M, Zou H. 2007. Development of efficient protein extraction methods for shotgun proteome analysis of formalin-fixed tissues. J Proteome Res. 6:1038-1047.

Kaczmarek E, Górna A, Majewski P. 2004. Techniques of image analysis for quantitative immunohistochemistry. Rocz Akad Med Bialymst. 49(suppl 1):155-158.

Kaczmarek E, Lacka K, Majewski P, Trzeciak P, Gorna A, Jarmolowska-Jurczyszyn D, Kluk A. 2008. Selected markers of proliferation and apoptosis in the parathyroid lesions: a spatial visualization and quantification. J Mol Hist. 39:509-517.

Kakimoto K, Takekoshi S, Miyajima K, Osamura RY. 2008. Hypothesis for the mechanism for heat-induced antigen retrieval occurring on fresh frozen sections without formalin-fixation in immunohistochemistry. J Mol Hist. 39:389-399.

Kitamoto T, Ogomori K, Tateishi J, Prusiner SB. 1987. Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. Lab Invest. 57:230-236.

Kitayama Y, Igarashi H, Sugimura H. 1999. Amplification of FISH signals using intermittent microwave irradiation for analysis of chromosomal instability in gastric cancer. Mol Pathol. 52:357-359.

Koshiba M, Ogawa K, Hamazaki S, Sugiyama T, Ogawa O, Kitajima T. 1993. The effect of formalin fixation on DNA and the extraction of high-molecular-weight DNA from fixed and embedded tissues. Pathol Res Pract. 189:66-72.

Krafft AE, Duncan BW, Bijwaard KE, Taubenberger JK, Lichy JH. 1997. Optimization of the isolation and amplification of RNA from formalin-fixed, paraffin-embedded tissue: the Armed Force Institute of Pathology experience and literature review. Mol Diagn. 2:217-230.

Kroll J, Becker KF, Kuphal S, Hein R, Hofstädter F, Bosserhoff AK. 2008. Isolation of high quality protein samples from punches of formalin fixed and paraffin embedded tissue blocks. Histol Histopathol. 23:391-395.

Kulkarni M, Prabhudesai N, Desai S, Borges A. 2000. Scrape cell-block technique for fine needle aspiration cytology smears. Cytopathology. 11:179-184.

Lan HY, Mu W, Ng YY, Nilolic-Paterson DJ, Atkins RC. 1996. A simple, reliable, and sensitive method for nonradioactive in situ hybridization: use of microwave heating to improve hybridization efficiency and preserve tissue morphology. J Histochem Cytochem. 44:281-287.

Lan HY, Mu W, Nikolic-Paterson DJ, Atkins RC. 1995. A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: use of microwave oven heating to block antibody crossreactivity and retrieve antigens. J Histochem Cytochem. 43:97-102.

Larsson L-I. 1988. Immunocytochemistry: theory and practice. Boca Raton (FL): CRC Press.

Leong AS-Y. 2009. Quantitative immunohistology: tissue section thickness, another glitch in the path to standardization. Appl Immunohistochem Mol Morphol. 17:465-469.

Liu H, Huang X, Zhang Y, Ye H, El Hamidi A, Kocjan G, Dogan A, Isaacson PG, Du M-Q. 2002. Archival fixed histologic and cytologic specimens including stained and unstained materials are amenable to RT-PCR. Diagn Mol Pathol. 11:222-227.

Liu H, Shi J, Wilkerson M, Huang Y, Meschter S, Dupree W, Lin F. 2007. Immunohistochemical detection of p16INK4a in liquid-based cytology specimens on cell block sections. Cancer. 111:74-82.

Liu J, Farhood A. 2004. Immunostaining for thyroid transcription factor-1 on fine-needle aspiration specimens of lung tumors: a comparison of direct smears and cell block preparations. Cancer. 102:109-114.

Long DJ, Buggs C. 2008. Microwave oven-based technique for immunofluorescent staining of paraffin-embedded tissues. J Mol Hist. 39:1-4.

Lucassen PJ, Labat-Moleur F, Negoescu A, van Lookeren Campagne M. 2000. Microwave-enhanced in situ end-labeling of apoptotic cells in tissue sections: pitfalls and possibilities. In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 71-91.

MacGrogan G, Soubeyran I, De Mascarel I, Wafflart J, Bonichon F, Durand M, Avril A, Mauriac L, Trojani M, Coindre JM.

1996. Immunohistochemical detection of progesterone receptors in breast invasive ductal carcinomas. Appl Immunohistochem. 4:219-227.

Mason JT, Fowler CB, O'Leary TJ. 2010. Study of formalin-fixation and heat-induced antigen retrieval. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 253-285.

Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. 1999. Analysis of chemical modificaiton of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res. 27:4436-4443.

Matkowskyj KA, Cox R, Jensen RT, Benya RV. 2003. Quantitative immunohistochemistry by measuring cumulative signal strength accurately measures receptor number. J Histochem Cytochem. 51:205-214.

McGhee JD, von Hippel PH. 1977a. Formaldehyde as a probe of DNA structure: 3. Equilibrium denaturation of DNA and synthetic polynucleotides. Biochemistry. 16:3267-3275.

McGhee JD, von Hippel PH. 1977b. Formaldehyde as a probe of DNA structure: 4. Mechanism of the initial reaction of formaldehyde with DNA. Biochemistry. 16:3276-3293.

McMahon J, McQuaid S. 1996. The use of microwave irradiation as a pretreatment to in situ hybridization for the detection of measles virus and chicken anaemia virus in formalin-fixed paraffin-embedded tissue. Histochem J. 28:157-164.

Mikhitarian K, Reott S, Hoover L, Allen A, Cole DJ, Gillanders WE, Mitas M. 2004. Enhanced detection of RNA from paraffin-embedded tissue using a panel of truncated gene-specific primers for reverse transcription. BioTechniques. 36:474-478.

Miller R, Kubier P. 2002. Immunohistochemistry on cytologic specimens and previously stained slides (when no paraffin block is available). J Histotechnol. 25:251-257.

Moelans CB, Kibbelaar RE, van den Heuvel MC, Castigliego D, de Weger RA, van Diest PJ. 2010. Validation of a fully automated HER2 staining kit in breast cancer. Cell Oncol. 32:149-155.

Moon Y, Park G, Han K, Kang C-S, Lee W. 2008. Mouse spleen tissue as a staining intensity reference for immunohistochemistry. Ann Clin Lab Sci. 38:215-220.

Mukai M, Setou M. 2010. Visualizing protein maps in tissue. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 369-389.

Murphy CL, Eulitz M, Hrncic R, Sletten K, Westermark P, Williams T, Macy SD, Wooliver C, Wall J, Weiss DT, et al. 2001. Chemical typing of amyloid protein contained in formalin-fixed paraffin-embedded biopsy specimens. Am J Clin Pathol. 116:135-142.

Nakamura KC, Kameda H, Koshimizu Y, Yanagawa Y, Kaneko T. 2008. Production and histological application of affinity-purified antibodies to heat-denatured green fluorescent protein. J Histochem Cytochem. 56:647-657.

Nakane PK, Pierce GBJ. 1966. Enzyme-labeled antibodies: preparation and application for the localization of antigens. J Histochem Cytochem. 14:929-931.

Niki H, Hosokawa S, Nagaike K, Tagawa T. 2004. A new immunofluorostaining method using red fluorescence of PerCP on formalin-fixed paraffin-embedded tissues. J Immunol Methods. 293:143-151.

Nirmalan NJ, Harnden P, Selby PJ, Banks RE. 2009. Development and validation of a novel protein extraction methodology for quantitation of protein expression in formalin-fixed paraffin-embedded tissues using Western blotting. J Pathol. 217:497-506.

O'Leary TJ, Fowler CB, Evers DL, Cunningham RE, Mason JT. 2010. Commentary: future directions. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 323-331.

O'Leary TJ, Fowler CB, Evers DL, Mason JT. 2009. Protein fixation and antigen retrieval: chemical studies. Biotech Histochem. 84:217-221.

Oliver KR, Heavens RP, Sirinathsinghji DJS. 1997. Quantitative comparison of pretreatment regimens used to sensitive in situ hybridization using oligonucleotide probes on paraffin-embedded brain tissue. J Histochem Cytochem. 45:1707-1713.

Ono A, Kumai T, Koizumi H, Nishikawa H, Kobayashi S, Tadokoro M. 2009. Overexpression of heat shock protein 27 in squamous cell carcinoma of the uterine cervix: a proteomic analysis using archival formalin-fixed, paraffin-embedded tissues. Hum Pathol. 40:41-49.

Palmer-Toy DE, Krastins B, Sarracino DA, Nadol JB Jr, Merchant SN. 2005. Efficient method for the proteomic analysis of fixed and embedded tissues. J Proteome Res. 4:2404-2411.

Pertschuk LP, Axiotis CA. 2000. Antigen retrieval for detection of steroid hormone receptors. In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 153-164.

Pinkus GS. 1982. Diagnostic immunocytochemistry of paraffin-embedded tissues. Hum Pathol. 13:411-415.

Press MF, Pike MC, Chazin VR, Hung G, Udove JA, Markowicz M, Danyluk J, Godolphin W, Sliwkowski M, Akita R, et al. 1993. Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res. 53:4971-4977.

Prieto DA, Hood BL, Darfler MM, Guiel TG, Lucas DA, Conrads TP, Veenstra TD, Krizman DB. 2005. Liquid Tissue™: proteomic profiling of formalin-fixed tissues. BioTechniques. 38:S32-S35.

Quach N, Myron GF, Shibata D. 2004. In vitro mutation artifacts after formalin fixation and error prone translesion synthesis during PCR. BMC Clin Pathol. 4:1.

Rahimi F, Shepherd CE, Halliday GM, Geczy CL, Raftery MJ. 2006. Antigen-epitope retrieval to facilitate proteomic analysis of formalin-fixed archival brain tissue. Anal Chem. 78:7216-7221.

Rait VK, O'Leary TJ, Mason JT. 2004. Modeling formalin fixatin and antigen retrieval with bovine pancreatic ribonuclease A: I. Structural and functional alterations. Lab Invest. 84:292-299.

Rait VK, Xu L, O'Leary TJ, Mason JT. 2004. Modeling formalin fixation and antigen retrieval with bovine pancreatic RBase A: II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. Lab Invest. 84:300-306.

Ramos-Vara JA. 2005. Technical aspects of immunohistochemistry. Vet Pathol. 42:405-426.

Redkar AA, Krishan A. 1999. Flow cytometric analysis of estrogen, progesterone receptor expression and DNA content in formalin-fixed, paraffin-embedded human breast tumors. Cytometry. 38:61-69.

Rhodes A, Jasani B, Anderson E, Dodson AR, Balaton AJ. 2002. Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: a comparative study involving results from laboratories in 21 countries. Am J Clin Pathol. 118:408-417.

Rhodes A, Jasani B, Balaton AJ, Miller KD. 2000. Immunohistochemical demonstration of oestrogen and progesterone receptors: correlation of standards achieved on in house tumours with that achieved on external quality assessment material in over 150 laboratories from 26 countries. J Clin Pathol. 53:292-301.

Riera J, Simpson JF, Tamayo R, Battifora H. 1999. Use of cultured cells as a control for quantitative immunocytochemical analysis of estrogen receptor in breast cancer: the Quicgel method. Am J Clin Pathol. 111:329-335.

Rimm DL. 2006. What brown cannot do for you. Nat Biotechnol. 24:914-916.

Robertson D, Savage K, Reis-Filho JS, Isacke CM. 2008. Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. BMC Cell Biol. 9:13.

Ronci M, Bonanno E, Colantoni A, Pieroni L, Di Ilio C, Spagnoli LG, Federici G, Urbani A. 2008. Protein unlocking procedures of formalin-fixed paraffin-embedded tissues: application to MALDI-TOF imaging MS investigations. Proteomics. 8:3702-3714.

Rupp GM, Locker J. 1988. Purification and analysis of RNA from paraffin-embedded tissues. Biotechniques. 6:56-60.

Saravanan RS, Rose JKC. 2004. A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. Proteomics. 4:2522-2532.

Sato Y, Sugie R, Tsuchiya B, Kameya T, Natori M, Mukai K. 2001. Comparison of the DNA extraction methods for polymerase chain reaction amplification from formalin-fixed and paraffin-embedded tissues. Diag Mol Pathol. 10:265-271.

Sherman M, Jimenez-Joseph D, Gangi MD, Rojas-Corona RR. 1994. Immunostaining of small cytologic specimens: facilitation with cell transfer. Acta Cytol. 1994:18-22.

Shi S-R, Balgley BM, Taylor CR. 2010. Symbiosis of immunohistochemistry and proteomics: marching to a new era. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 391-397.

Shi S-R, Cote RJ, Chaiwun B, Young LL, Shi Y, Hawes D, Chen T, Taylor CR. 1998. Standardization of immunohistochemistry based on antigen retrieval technique for routine formalin-fixed tissue sections. Appl Immunohistochem. 6:89-96.

Shi S-R, Cote C, Kalra KL, Taylor CR, Tandon AK. 1992. A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. J Histochem Cytochem. 40:787-792.

Shi S-R, Cote RJ, Shi Y, Taylor CR. 2000. Antigen retrieval technique. In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 311-333.

Shi S-R, Cote RJ, Taylor CR. 1997. Antigen retrieval immunohistochemistry: past, present, and future. J Histochem Cytochem. 45:327-343.

Shi S-R, Cote RJ, Taylor CR. 1999. Standardization and further development of antigen retrieval immunohistochemistry: strategies and future goals. J Histotechnol. 22:177-192.

Shi S-R, Cote RJ, Taylor CR. 2000. Antigen retrieval immunohistochemistry used for routinely processed celloidin-embedded human temporal bone sections. In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 287-307.

Shi S-R, Cote RJ, Taylor CR. 2001. Antigen retrieval immunohistochemistry and molecular morphology in the year 2001. Appl Immunohistochem Mol Morphol. 9:107-116.

Shi S-R, Cote RJ, Wu L, Liu C, Datar R, Shi Y, Liu D, Lim H, Taylor CR. 2002. DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: heating under the influence of pH. J Histochem Cytochem. 50:1005-1011.

Shi S-R, Datar R, Liu C, Wu L, Zhang Z, Cote RJ, Taylor CR. 2004. DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. Histochem Cell Biol. 122:211-218.

Shi S-R, Gu J, Cote RJ, Taylor CR. 2000. Standardization of routine immunohistochemistry: where to begin? In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 255-272.

Shi S-R, Gu J, Kalra KL, Chen T, Cote RJ, Taylor CR. 1995. Antigen retrieval technique: a novel approach to immunohistochemistry on routinely processed tissue sections. Cell Vision. 2:6-22.

Shi S-R, Gu J, Taylor CR, editors. 2000. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton.

Shi S-R, Gu J, Turrens F, Cote RJ, Taylor CR. 2000. Development of the antigen retrieval technique: philosophy and theoretical base. In: Shi S-R, Gu J, Taylor CR, editors. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick (MA): Eaton. p. 17-39.

Shi S-R, Key ME, Kalra KL. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 39:741-748.

Shi S-R, Liu C, Balgley BM, Lee C, Taylor CR. 2006. Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. J Histochem Cytochem. 54:739-743.

Shi S-R, Liu C, Perez J, Taylor CR. 2005. Protein-embedding technique: a potential approach to standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections. J Histochem Cytochem. 53:1167-1170.

Shi S-R, Liu C, Pootrakul L, Tang L, Young A, Chen R, Cote RJ, Taylor CR. 2008. Evaluation of the value of frozen tissue section used as "gold standard" for immunohistochemistry. Am J Clin Pathol. 129:358-366.

Shi S-R, Liu C, Taylor CR. 2007. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval technique: from experiments to hypothesis. J Histochem Cytochem. 55:105-109.

Shi S-R, Roth KA, Taylor CR. 2010. Key issues and strategies of standardization for quantifiable immunohistochemistry. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 75-86.

Shi S-R, Taylor CR. 2010a. Extended application of antigen retrieval technique in immunohistochemistry and in situ hybridization. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 25-45.

Shi S-R, Taylor CR, editors. 2010b. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley.

Shi S-R, Taylor CR. 2010c. Extraction of DNA/RNA from formalin-fixed, paraffin-embedded tissue based on the antigen retrieval principle. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 47-71.

Shi S-R, Taylor CR. 2010d. Preface. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. ix-xiv.

Shi S-R, Taylor CR. 2010e. Standardization of antigen retrieval techniques based on the test battery approach. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 3-24.

Shi S-R, Taylor CR. 2010f. Tissue cell sample preparation: lessons from the antigen retrieval technique. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 189-194.

Shi Y, Wasserman PG. 2010. Cell sample preparation for clinical cytopathology: current status and future development. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 219-234.

Shibata D, Martin WJ, Arnheim N. 1988. Analysis of DNA sequences in forty-year-old paraffin-embedded thin-tissue sections: a bridge between molecular biology and classical histology. Cancer Res. 48:4564-4566.

Shidham VB, Chang C-C, Rao RN, Komorowski R, Chivukula M. 2003. Immunostaining of cytology smears: a comparative study to identify the most suitable method of smear preparation and fixation with reference to commonly used immunomarkers. Diagn Cytopathol. 29:217-221.

Shidham VB, Lindholm PF, Kajdacsy-Balla A, Chang CC, Komorowski R. 2000. Methods of cytologic smear preparation and fixation: effect on the immunoreactivity of commonly used anticytokeratin antibody AE1/AE3. Acta Cytol. 44:1015-1022.

Shin SJ, Chen B, Hyjek E, Vazquez M. 2007. Immunocytochemistry and fluorescence in situ hybridization in Her-2/neu status in cell block preparations. Acta Cytol. 51:552-557.

Shiurba RA, Spooner ET, Ishiguro K, Takahashi M, Yoshida R, Wheelock TR, Imahori K, Cataldo AM, Nixon RA. 1998. Immunocytochemistry of formalin-fixed human brain tissues: microwave irradiation of free-floating sections. Brain Res Brain Res Protoc. 2:109-119.

Shroyer KR, Homer P, Heinz D, Singh M. 2006. Validation of a novel immunocytochemical assay for topoisomerase II- and minichromosome maintenance protein 2 expression in cervical cytology. Cancer. 108:324-330.

Shtilbans V, Szporn AH, Wu M, Burstein DE. 2005. p63 immunostaining in destained bronchoscopic cytological specimens. Diagn Cytopathol. 32:198-203.

Sibony M, Commo F, Callard P, Gasc J-M. 1995. Enhancement of mRNA in situ hybridization signal by microwave heating. Lab Invest. 73:586-591.

Sompuram AR, Vani K, Messana E, Bogen SA. 2004. A molecular mechanism of formalin fixation and antigen retrieval. Am J Clin Pathol. 121:190-199.

Sompuram SR, Vani K, Bogen SA. 2006. A molecular model of antigen retrieval using a peptide array. Am J Clin Pathol. 125:91-98.

Sprung RWJ, Brock JW, Tanksley JP, Li M, Washington MK, Slebos RJ, Liebler DC. 2009. Equivalence of protein inventories obtained from formalin-fixed paraffin-embedded and frozen tissue in multidimensional liquid chromatography–tandem mass spectrometry shotgun proteomic analysis. Mol Cell Proteomics. 8:1988-1998.

Stirling JW, Graff PS. 1995. Antigen unmasking for immunoelectron microscopy: labeling is improved by treating with sodium ethoxide or sodium metaperiodate, then heating on retrieval medium. J Histochem Cytochem. 43:115-123.

Strater J, Gunthert AR, Bruderlein S, Moller P. 1995. Microwave irradiation of paraffin-embedded tissue sensitizes the TUNEL method for in situ detection of apoptotic cells. Histochemistry. 103:157-160.

Suthipintawong C, Leong AS-Y, Chan K-W, Vinyuvat S. 1997. Immunostaining of estrogen receptor, progesterone receptor, MIB1 antigen, and c-erbB-2 oncoprotein in cytologic specimens: a simplified method with formalin fixation. Diagn Cytopathol. 17:127-133.

Suthipintawong C, Leong AS-Y, Vinyuvat S. 1996. Immunostaining of cell preparations: a comparative evaluation of common fixatives and protocols. Diagn Cytopathol. 15:167-174.

Taylor CR. 1979. Immunohistologic studies of lymphomas: new methodology yields new information and poses new problems. J Histochem Cytochem. 27:1189-1191.

Taylor CR. 1980. Immunohistologic studies of lymphoma: past, present and future. J Histochem Cytochem. 28:777-787.

Taylor CR. 2001. Immunohistochemistry for the age of molecular morphology. Appl Immunohistochem Mol Morphol. 9:1-2.

Taylor CR. 2006a. Quantifiable internal reference standards for immunohistochemistry: the measurement of quantity by weight. Appl Immunohistochem Mol Morphol. 14:253-259.

Taylor CR. 2006b. Standardization in immunohistochemistry: the role of antigen retrieval in molecular morphology. Biotech Histochem. 81:3-12.

Taylor CR, Burns J. 1974. The demonstration of plasma cells and other immunoglobulin containing cells in formalin-fixed, paraffin-embedded tissues using peroxidase labelled antibody. J Clin Pathol. 27:14-20.

Taylor CR, Cote RJ. 2005. Immunomicroscopy: a diagnostic tool for the surgical pathologist. Philadelphia: Elsevier Saunders.

Taylor CR, Kledzik G. 1981. Immunohistologic techniques in surgical pathology: a spectrum of new special stains. Hum Pathol. 12:590-596.

Taylor CR, Levenson RM. 2006. Quantification of immunohistochemistry: issues concerning methods, utility and semiquantitative assessment II. Histopathology. 49:411-424.

Taylor CR, Mason DY. 1974. The immunohistological detection of intracellular immunoglobulin in formalin-paraffin sections from multiple myeloma and related conditions using the immunoperoxidase technique. Clin Exp Immunol. 18:417-429.

Taylor CR, Shi S-R, Barr NJ. 2010. Techniques of Immunohistochemistry: principles, pitfalls, and standardization. In: Dabbs DJ, editor. Diagnostic immunohistochemistry: theranostic and genomic applications. 3rd ed. Philadelphia: Saunders-Elsevier. p. 1-41.

Torlakovic EE, Riddell R, Banerjee D, El-Zimaity H, Pilavdzic D, Dawe P, Magliocco A, Barnes P, Berendt R, Cook D, et al. 2010. Canadian Association of Pathologists–Association canadienne des pathologistes National Standards Committee/ Immunohistochemistry: best practice recommendations for standardization of immunohistochemistry tests. Am J Clin Pathol. 133:354-365.

van der Ploeg M, Duijndam WAL. 1986. Matrix models: essential tools for microscopic cytochemical research. Histochemistry. 84:283-300.

Verbeek DH, Smedts F, Wijnen-Dubbers CW, Mravunac M. 1996. Histologic processing of thick tissue specimens from cytology slides. Acta Cytol. 40:1198-1204.

Viegas MS, Martins TC, Seco F, do Carmo A. 2007. An improved and cost-effective methodology for the reduction of autofluorescence in direct immunofluorescence studies on formalin-fixed paraffin-embedded tissues. Eur J Histochem. 51:59-66.

Weintraub J, Redard M, Wenger D, Vassilakos P. 1990. The application of immunocytochemical techniques to routinely-fixed and stained cytologic specimens: an aid in the differential diagnosis of undifferentiated malignant neoplasms. Pathol Res Pract. 186:658-665.

Wick MR, Mills S. 2001. Consensual interpretive guidelines for diagnostic immunohistochemistry. Am J Surg Pathol. 25:1208-1210.

Williams C, Pontén F, Moberg C, Söderkvist P, Uhlén M, Pontén J, Sitbon G, Lundeberg J. 1999. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol. 155:1467-1471.

Wilson DF, Jiang D-J, Pierce AM, Wiebkin OW. 1996. Antigen retrieval for electron microscopy using a microwave technique for epithelial and basal lamina antigens. Appl Immunohistochem. 4:66-71.

Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, et al. 2007. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med. 131:18-43.

Xia X, Rasmussen T, Alvarez X, Taguchi T, Li M, La Russa VF. 2007. Fluorescence in situ hybridization using an old world monkey Y chromosome-specific probe combined with immunofluorescence staining on rhesus monkey tissues. J Histochem Cytochem. 55:1115-1121.

Xu H, Yang L, Wang W, Shi S-R, Liu C, Liu Y, Fang X, Taylor CR, Lee CS, Balgley BM. 2008. Antigen retrieval for proteomic characterization of formalin-fixed and paraffin-embedded tissues. J Proteome Res. 7:1098-1108.

Yamashita S. 2007. Heat-induced antigen retrieval: mechanisms and application to histochemistry. Prog Histochem Cytochem. 41:141-200.

Yamashita S. 2010. pH or ionic strength of the antigen retrieval solution: a potential role for refolding during heating treatment. In: Shi S-R, Taylor CR, editors. Antigen retrieval immunohistochemistry based research and diagnostics. Hoboken (NJ): John Wiley. p. 303-321.

Yamashita S, Okada Y. 2005a. Application of heat-induced antigen retrieval to aldehyde-fixed fresh frozen sections. J Histochem Cytochem. 53:1421-1432.

Yamashita S, Okada Y. 2005b. Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. J Histochem Cytochem. 53: 13-21.

Yoshida T, Sano T, Kanuma T, Owada N, Sakurai S, Fukuda T, Nakajima T. 2008. Immunochemical analysis of HPV L1 capsid protein and p16 protein in liquid-based cytology samples from uterine cervical lesions. Cancer. 114:83-88.