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Nile Red Staining as a Sentinel Screen for Phospholipids

Diane L. Pecht, Deborah A. Litz, Carla L. Bregman, Stephen K. Durham, Mark G. Mense
Departments of Pathology and Drug Safety Evaluation
Bristol-Myers Squibb Company
Syracuse, NY

diane.pecht@bms.com

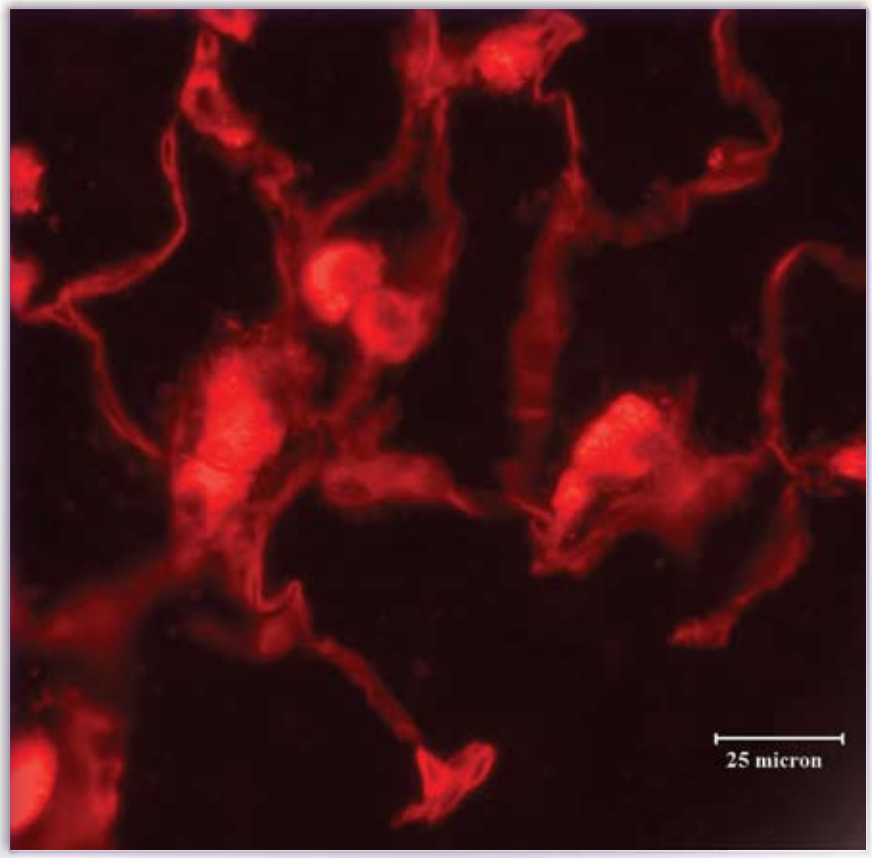

Fig. 1. Formalin-fixed frozen section of rat lung tissue stained with Nile red. Lung tissue came from rat given high doses of a test substance. The phospholipids appear as bright hot spots, orange-red in color. 400X

Introduction

Nile red, a fluorescent hydrophobic dye, was chosen to demonstrate the presence of phospholipids in frozen sections of lungs from drug-treated rats used in a 2-week toxicology study. Electron microscopy performed on rat lung tissue collected during the study showed the presence of membranous lamellar (multilamellar), or myelinoid, bodies compatible with those observed with phospholipidosis. Based on these findings, Nile red staining can be used as a simple, rapid, and inexpensive sentinel screen for phospholipids.

Materials and Methods

A test substance was administered daily by gavage to Sprague-Dawley rats for 2 weeks. At the end of dosing, selected tissues were collected at necropsy and fixed in 10% neutral buffered formalin.

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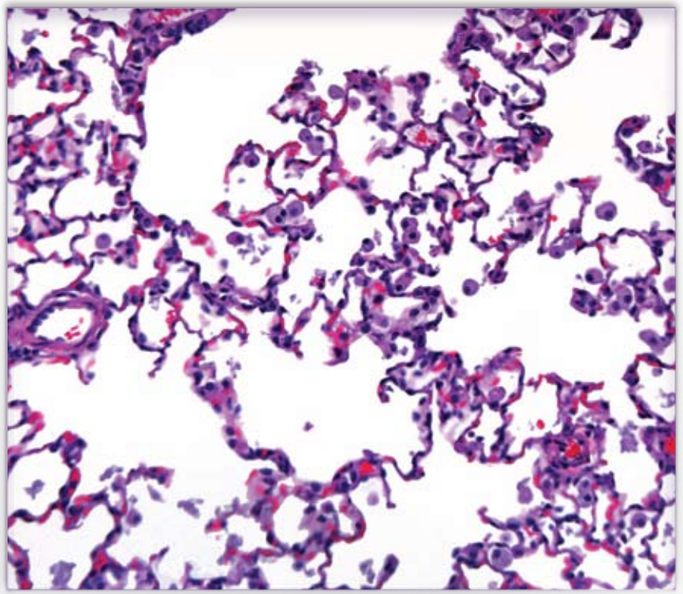

Fig. 2. Microscopic examination of an H&E-stained section of lung from a high-dose rat. The formalin-fixed, paraffin-embedded lung revealed increased numbers of highly vacuolated macrophages in the pulmonary alveoli. 100X

During the light microscopic evaluation of hematoxylin and eosin (H&E)-stained, paraffin-embedded lung sections, increased numbers of highly vacuolated macrophages were observed in the pulmonary alveoli of drug-treated animals.

Additional stains such as oil-red O and Nile red were used to further characterize the vacuolar contents of macrophages in the lungs of high-dose rats. Lung sections from vehicle-treated rats served as controls. For the Nile red staining procedure, formalin-fixed sections of lungs were embedded in Tissue-Tek® O.C.T. (optimal cutting temperature) compound (Sakura Finetek, Torrance, CA), sectioned at 4-5 µm in a cryostat, and stained under darkened conditions with a Nile red solution. The sections were then examined with a Nikon fluorescent microscope using a rhodamine filter (wavelength ~540 nm).

The finding of phospholipids was further confirmed by electron microscopy of highly vacuolated pulmonary macrophages. Membranous lamellar (myelinoid bodies), which were consistent with phospholipidosis, were observed.

Staining

Nile red (9-diethylamino-5H-benzo[α]-phenoxazine-5-one) is a phenoxazone dye that fluoresces intensely, and in a range of colors, when in contact with organic solvents and hydrophobic lipids.[1] The dye has been used to distinguish between normal cells and cells containing lysosomal accumulations of phospholipids. The color of the observed fluorescence is directly dependent on the hydrophobicity of the surrounding environment. This unique property of Nile red allows for the staining of both neutral lipids (yellow-gold color) and phospholipids (orange-red color), when using an appropriate filter package.[2]

A Nile red stock solution (500 µg/mL) was prepared in acetone and diluted with 75% aqueous glycerol to a concentration of 2.5 µg/mL.[3]

The following procedure was used to stain frozen lung sections to demonstrate phospholipids:

1. Section frozen tissue at 4-5 microns.
2. Rinse sections in deionized water for 1-2 minutes.
3. Place 1 drop of Nile red/glycerol staining solution onto the tissue and coverslip.
   Note: Handle Nile red solutions and perform staining in subdued lighting.
4. Stain for 5 minutes and examine slides using a fluorescent microscope.

Results

The light microscopic appearance of pulmonary histiocytosis, characterized by the presence of vacuolated macrophages within the alveoli, is presented in Figure 2. Figure 1 demonstrates a positive Nile red stain for the pulmonary histiocytes, indicating phospholipidosis as the underlying mechanism for the histiocytosis. The diagnosis of phospholipidosis is confirmed by the transmission electron microscopic evidence of accumulating multilamellar bodies within the histiocytes (Fig. 3).

Conclusion

Phospholipidosis is a lipid storage disorder in which excess phospholipids accumulate within cells. Some cationic, amphiphilic drugs are known to have the potential to induce production of phospholipids. This condition of phospholipidosis is characterized by an intracellular accumulation of phospholipids and the concurrent development of lamellar bodies. The primary mechanism responsible for the development of phospholipidosis is an inhibition of lysosomal phospholipase activity by the drug.

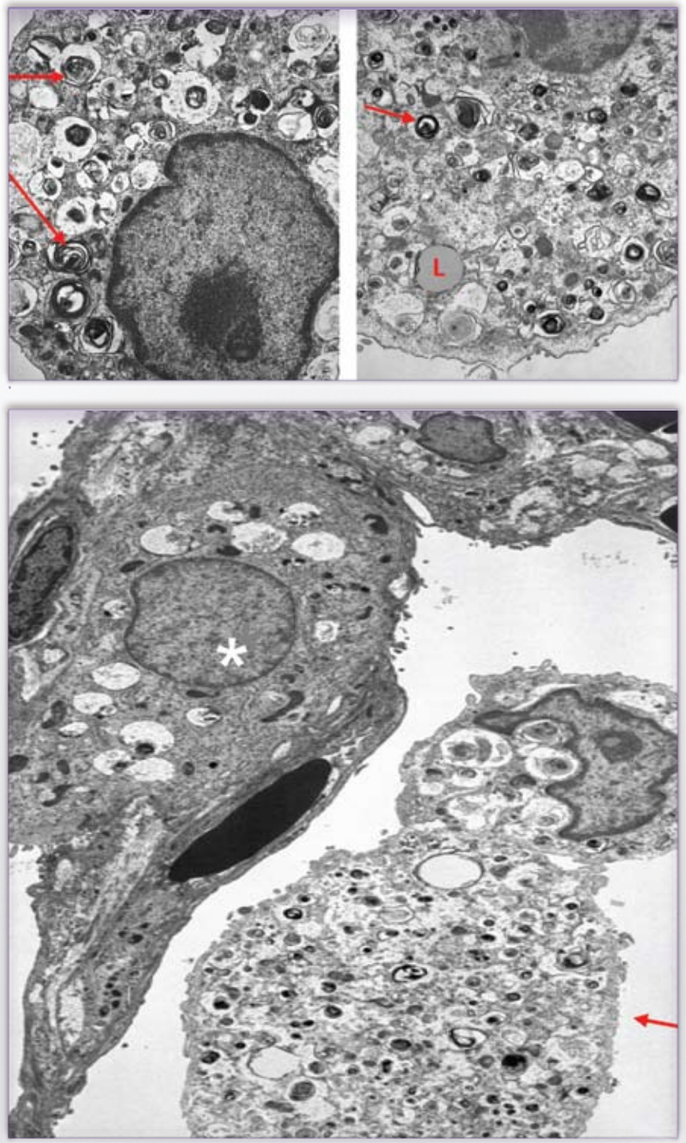

Fig. 3. Formalin-fixed, high-dose drug-treated rat lungs. Alveolar macrophages are filled with multilamellar bodies (red arrows) and an occasional lipid droplet (L). The white star denotes a nucleus of a pneumocyte.

In this study, electron microscopy demonstrated myelinoid bodies consistent with incomplete degradation of cell-derived phospholipids (the hallmark of phospholipidosis) in formalin-fixed sections of lung from drug-treated animals. Nile red staining of frozen lung sections from the same animals also demonstrated phagocytized phospholipids in foamy alveolar macrophages, but in a shorter time and with less expenditure than required with electron microscopy.

The Nile red staining method used in this study clearly demonstrated the presence of phospholipids. This method proved to be a simple, rapid, and inexpensive procedure that can be used as a sentinel screen for phospholipids in future studies where drug-induced phospholipidosis is suspected. Electron microscopy and other special stains may then be requested to further confirm this finding.

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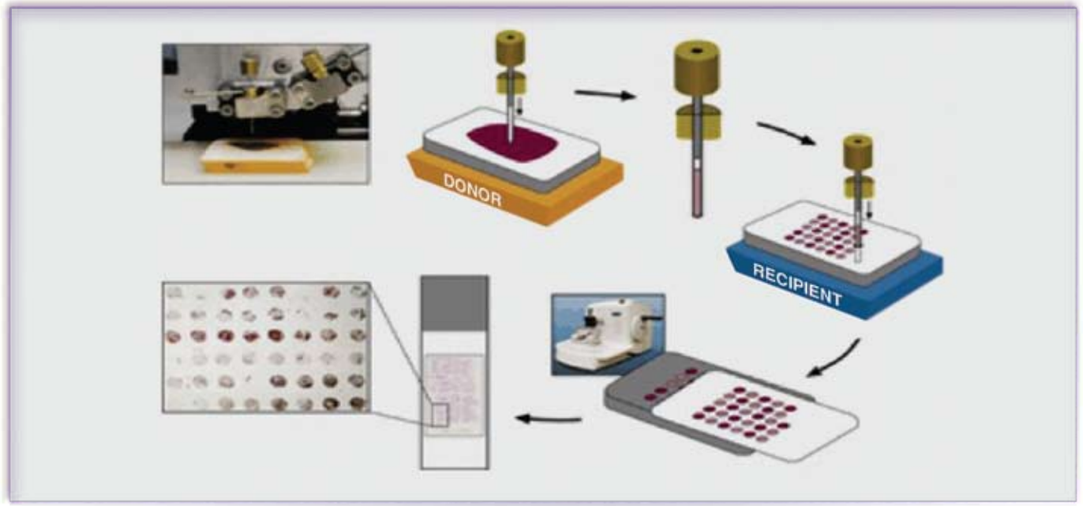

Fig. 1. The technique used to prepare the array control block of these selected connective tissues.

Fig. 2. The TMA control block, prepared slides, and microscopic images demonstrating basement membranes of kidney stained with PAMS and PAS.

Tissue Microarray Controls to Demonstrate Connective Tissue Components

Yan Cai,* Zhongqiu Guo,*
Aruna Kola,* Hazel Dalton,*
Mark Bailey,* and Sharon LeBlanc†

***University of Texas**
M.D. Anderson Cancer Center
School of Health Sciences
Program in Histotechnology

†Baylor College of Medicine
Breast Center
Houston, Texas

cyzhangus@yahoo.com

Introduction

The tissue microarray (TMA) procedure is an important new innovative approach in the preparation of paraffin tissue blocks that are used as control slides for histologic special staining techniques. It is especially useful for light microscopy applications of special stains utilizing a few sample cores. This article demonstrates a procedure using tissue microarray technology that was described by J. Quinonez and S. Magedson on April 1, 2003, in a lecture entitled "Tissue Microarrayer: Principles, Procedures and Applications."

We prepared control slides for light microscopy special staining techniques to demonstrate various connective tissue components. The special stains used were Masson trichrome to differentiate collagen and muscle; phosphotungstic acid hematoxylin (PTAH) to demonstrate striated muscle; silver stains (Snook's) to demonstrate reticulum fibers; Verhoeff-van Gieson (VVG) to demonstrate elastic fibers; Jones' periodic acid methenamine silver (PAMS) and periodic acid-Schiff (PAS) to demonstrate basement membranes.

An article published by Rimm et al[1] states that TMA is a new and innovative technology offering several advantages. In our studies, the advantages include enabling pathologists and histotechnologists to examine multiple samples that are arranged on a single slide instead of analyzing tissue samples one slide at a time. This one slide with multiple tissue cores is sufficient to use when staining for any one of the connective tissue components requested. Another important advantage is that because only a small piece of tissue is needed for this type of analysis, TMA is instrumental in preserving tissue samples.

Materials and Methods

For this study, the Beecher Tissue Manual Microarrayer (Beecher Instruments, Inc., Sun Prairie, WI) was used. Normal rat and mouse tissue from spleen, liver, kidney, and lymph nodes was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4-6 microns. Initially, tissue sections were stained for light microscopy identification of the connective tissues in order to identify areas where cores of tissue containing sufficient connective tissue could be removed from each donor block and placed in a recipient block to be used for controls. Slides were examined microscopically and areas were identified and marked. The technique used to prepare the array control block of these selected connective tissues is detailed in Figure 1. Cores of tissues containing sufficient connective tissue were removed from each donor block and placed into a recipient block utilizing a 1.5 mm stylet, and then precisely arrayed to a new block using the arrayer instrument. Multiple consecutive sections were cut from blocks and used as targets for staining with Masson trichrome, PTAH, Snook's, VVG, PAMS, and PAS.

Procedure

Formalin-fixed, paraffin-embedded control tissue blocks, known to be positive for connective tissue, are cut at 4-6 microns from each of the donor blocks before special stains are performed. A typical procedure follows the steps outlined below.

1. Design the array by organizing tissues in a manner that will best meet your needs.
2. Mark the area of interest on the last section cut from the donor block.
3. Place all blocks and slides in order according to the array design.
4. Prepare recipient block.
   - Use paraffin with a melting point of 55˚C-58˚C
   - The blank recipient block should be free from cracks and bubbles
   - Secure recipient block-to-block holder
5. Find starting area and reset the micrometers on the tissue arrayer to zero.
6. Start punching with desired stylet.

Results

The stained sections from the tissue array block result in high-quality control slides that can be utilized to demonstrate the selected connective tissue components. The 1.5 mm cores provide sufficient representation of collagen, reticulum fibers, elastic fibers, and basement membranes in the array block.

Discussion

For this study, the Beecher Tissue Manual Microarrayer was used, and stained slides were examined microscopically. Core areas with sufficient tissue were selected from each donor block and placed in the recipient block. These selected areas of tissue were for muscle, elastic, collagen, and reticulum fibers, as well as basement membranes. The tissue array block was designed by using 1.5 mm cores from each of the donor blocks. When used to prepare control slides to demonstrate these connective tissue components, the tissue array blocks offer several advantages. First, the usefulness of tissue blocks is extended for a much longer period. Second, more sections per slide can be obtained than by sectioning individual tissue blocks for each control block. Third, instead of preparing boxes of control slides from blocks of each individual connective tissue component, only one box of the tissue array control slides is produced from a single block. Irreplaceable archival materials can be preserved because of the small size of the cores used.

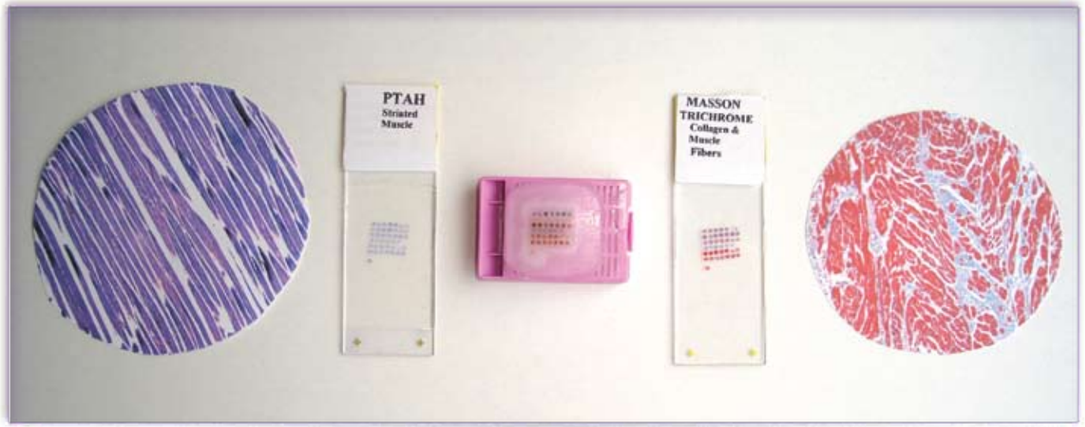

Fig. 3. The TMA control block, prepared slides, and a microscopic image demonstrating muscle striations in skeletal muscle stained with PTAH as well as a microscopic image demonstrating collagen and muscle in heart stained with Masson trichrome.

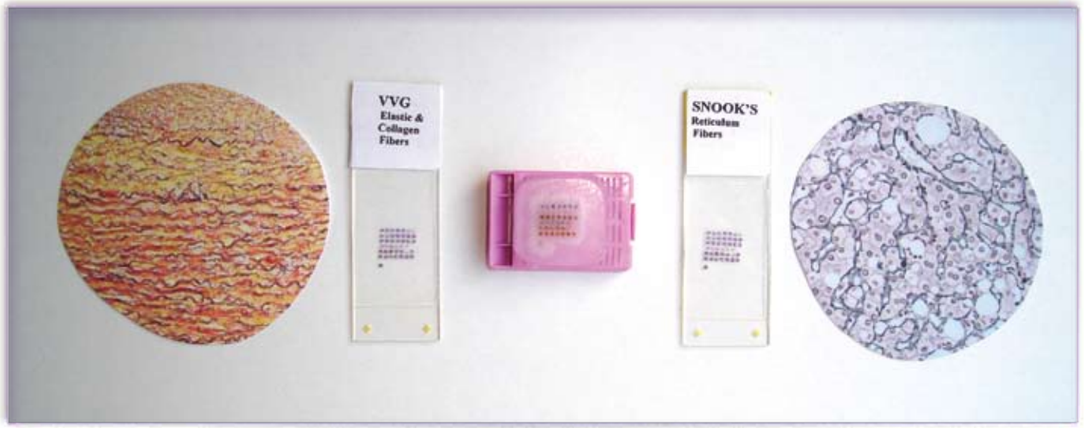

Fig. 4. The TMA control block, prepared slides, and microscopic image demonstrating elastic and collagen fibers in artery stained with VVG and reticulum fibers in liver stained with Snook's.

Conclusion

Microarray technology is a valuable tool when applied to designing tissue array control blocks. TMA control blocks can help histotechnologists demonstrate connective tissue components when using special staining techniques.

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Acknowledgments

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Technical Methodology in the Processing of Melanocytic Lesions in the Mohs Laboratory

Amanda P. Finney, BS
Carlette M. Geddis, BS, HTL (ASCP)
Medical University of South Carolina
Charleston, SC

finney@musc.edu

Traditionally, the histologic preparation of melanocytic lesions has been relegated to the surgical pathology laboratory; however, technological advances now allow for tissue processing within the Mohs laboratory. The margins of a melanocytic lesion can be determined from paraffin sections and by using immunohistochemistry on frozen sections. Technical issues of each methodology are of paramount importance when deciding which technique to employ.

Mohs micrographic surgery is a specialized treatment for the total surgical removal of skin cancer. This technique is designed to conserve normal tissue while eliminating the malignancy, and relies on the microscopic examination of each surgical layer until the margins are clear of tumor.

On the day of surgery, when the tissue is excised, the surgical margins are color coded using colored, insoluble inks. This allows the tissue to be mapped for orientation (Fig. 1).

The tissue is then given to the lab for the preparation of frozen sections. However, if a biopsy is suspected of being melanocytic in origin, the surgeon will decide whether to prepare the tissue using frozen sections or paraffin sections. Each surgical layer is examined microscopically and diagnosed by the Mohs surgeon who must decide if excision of another layer will be necessary. This process is repeated until all of the cancerous tissue has been excised. For very large lesions, surgery may continue for consecutive days until clear surgical margins are achieved.

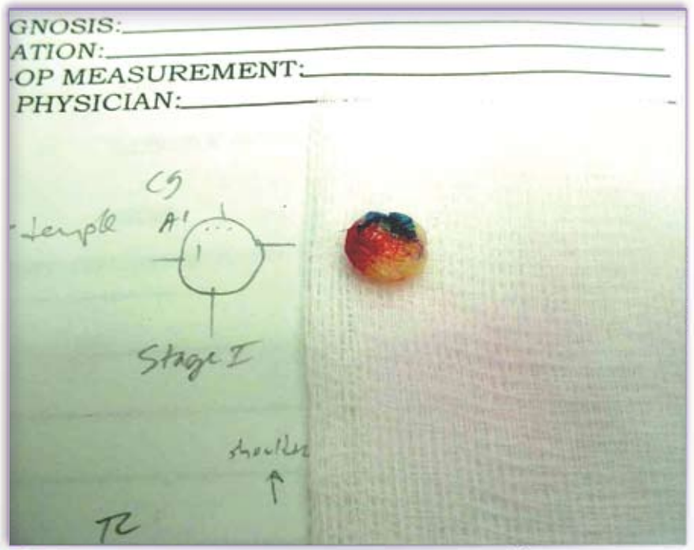

Fig. 1. Mohs surgical sample. Upon arrival at the lab, specimen must be correlated with the map drawn by the surgeon (left in photo) and marked with different colored inks to identify surgical margins.

Paraffin sectioning is the predominant method used in preparing microscope slides of melanocytic lesions. This technique requires the tissues to be chemically processed through a dehydrant (usually alcohol) and a paraffin solvent, which removes water and lipids so the specimen can be infiltrated with a paraffin supporting medium.[1] Proper fixation is the most critical step in preparing tissues for paraffin processing. Fixation time (duration) is key to achieving high-quality histologic sections and is often determined by the size and anatomic location of the lesion. The average fixation time for excised tissue on a closed system processor such as the Sakura Tissue-Tek® VIP® (Sakura Finetek, Torrance, CA) ranges from 6 to 8 hours for samples 4 mm thick or less. Once the lesion has been processed and embedded, the technologist will begin to section the specimen. Sections are usually cut between 4 and 6 microns and are then stained using hematoxylin and eosin (H&E).

The advantages and disadvantages of paraffin sections are well documented.[2] Cellular clarity is considerably enhanced in paraffin sections as individual cells are clearly visualized in H&E-stained paraffin sections, which makes it easier to distinguish different cell types. In addition, there is a large library of normal and abnormal tissues describing the appearance and visual characteristics demonstrated in paraffin sections. Some surgeons believe that lesion interpretation in permanent sections is worth the long turnaround time. Prolonged turnaround time, however, is a significant disadvantage of chemical processing. Unlike the rapid turnaround time associated with frozen section preparation of typical Mohs lesions, paraffin sections can take, on average, 24 hours to complete one surgical layer. This lengthy time frame is due to the need for adequate fixation and processing. This can pose a significant inconvenience for the patient, who must wait and remain available for more than 24 hours to find out if more surgical layers will be necessary on subsequent days, since permanent sections allow only one layer to be evaluated per 24-hour period.

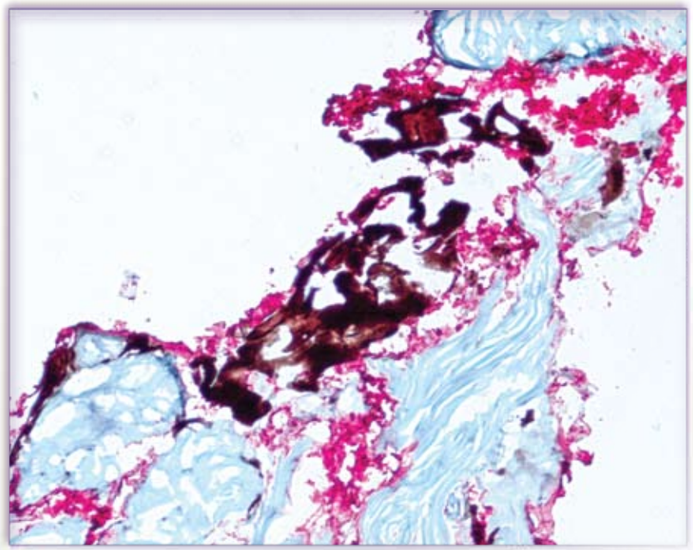

Fig. 2. Frozen section of the sample is stained with Mart-1 antibody using diaminobenzidine (DAB) chromogen. Mart-1 positive cells stain brown/black. Red is the ink used to mark the surgical margin. The appearance of Mart-1 positive tumor cells along the surgical margin indicates additional tissue must be excised in order to eliminate the melanoma. 200X

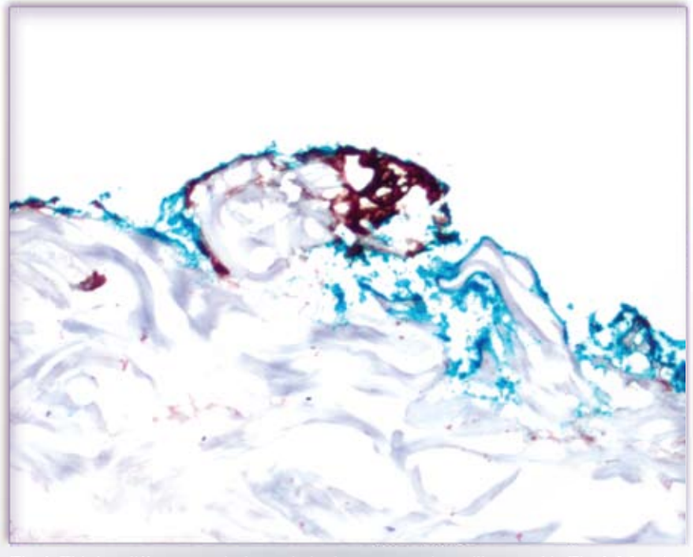

Fig. 3. This is a different surgical margin in the same specimen illustrated in Fig. 2. Mart-1 positive cells stain brown/black. Blue ink was used to mark this surgical margin. Additional Mart-1 positive staining indicates that further surgery is needed. 100X

Immunohistochemistry (IHC) performed on frozen sections is rapidly becoming the new diagnostic method for melanocytic lesions as diagnostic turnaround is much shorter than with paraffin sections. When the tissue arrives in the lab, the technologist embeds the tissue in the cryostat and cuts sections at 3 to 5 microns thick. The slides are then used in an IHC staining protocol that takes approximately 1 hour to complete (see Figs. 2 and 3 and Table 1). Antibodies used to detect melanomas in frozen sections include Mart-1/Melan A and Mel-5. The primary advantage of IHC staining of Mohs frozen sections is the relatively rapid turnaround time when compared to that of paraffin sections. IHC on frozen sections allows multiple surgical layers to be performed the same day, as the Mohs frozen sectioning and IHC staining can be completed in about 2 hours.

The high sensitivity of the Mart-1 antibody for detecting melanomas offers a significant advantage over H&E staining for the diagnosis of melanocytic lesions in frozen sections because the Mart-1 antibody is specific for one cell type. Individual melanocytes may be too subtle to view on frozen sections stained using H&E, and they may be difficult to distinguish from keratinocytes as well as melanocytic hyperplasia in sun-damaged skin. When Mart-l is used in conjunction with H&E-stained slides, it can be a useful tool in the rapid evaluation of melanoma.[3] Multiple layers can be taken in one day because of the relatively short turnaround time for IHC on frozen sections (2 to 4 hours). This time varies based on size and fat content of the tissue.

Table 1. Mart-1 Staining Protocol*

1. Cut epidermal sections at 3-4 microns and dermis sections at 8-10 microns
2. Place sections on charged slides labeled in the following order: 1. H&E – 2 sections 2. Mart-1 – 1 section 3. Mart-1 – 1 section 4. Immuno Negative – 1 section 5. H&E – 2 sections
3. Stain the H&E slides and allow the IHC slides to air dry for 5 minutes
4. IHC slides are placed into a 62°C oven to enhance tissue adherence to the slide – 5 minutes
5. Allow to cool and fix in acetone – 3 minutes
6. Air dry – 4 minutes
7. Rehydrate in Tris buffer (cat# S3001, Dako Corp., Carpinteria, CA) – 4 minutes
8. Apply Mart-1 (cat# MS716-R7, Lab Vision Corp., Fremont, CA) to patient slides and positive control tissue slide – incubate 10 minutes at room temperature in a humid chamber
9. Apply universal negative control serum (cat# N1698, Dako Corp.) to patient negative control section – incubate 10 minutes at room temperature in a humid chamber
10. Rinse in Tris buffer – 2 minutes
11. Apply Poly-HRP (cat# DPVM-110DAB, Immuno Vision, Springdale, AZ) – incubate 10 minutes at room temperature in a humid chamber
12. Rinse in Tris buffer – 2 minutes
13. Apply DAB chromogen† (cat# DPVM-110DAB, Immuno Vision) – incubate 2 minutes at room temperature in a humid chamber
14. Rinse in distilled water – 2 minutes
15. Counterstain with hematoxylin – 1 minute; dehydrate in reagent grade alcohol and mount

* Protocol modified from original provided to us by Duke University.
† DAB chromogen should be mixed during the 10 minute Poly-HRP.

There are pitfalls when performing IHC on frozen sections that can lead to variable staining results, which the technologist should be aware of. Since most Mohs labs do not have sufficient IHC volume to justify the expense of an automated IHC stainer, the staining protocol is often carried out manually. This can sometimes cause run-to-run staining variability, especially if more than one staff member performs the staining, as slight variations in technique can lead to noticeable staining results. When this occurs, it may be necessary to repeat the Mart-1 stain, which compromises the surgeon's ability to perform multiple surgical layers in the same day. Of course, the need to repeat the staining procedure increases usage of the costly IHC reagents—another disadvantage to the laboratory. Variability in section thickness may also contribute to staining variability. IHC should be carried out on sections that are cut between 3 and 5 microns. These thin sections may be hard for one to achieve, however, cutting sections too thick will make cells difficult to visualize.

In the ultimate care of their patients, both the surgeon and lab staff must weigh the advantages and disadvantages of each method in order to make an informed, strategic decision in choosing the optimal processing procedure for each individual case.

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Fig. 1. Wire stain racks and vinyl-coated lead wire prevent the containers from tipping over. The water bath is used at 98°C -100°C. Temperatures are monitored with a dial thermometer.

High Temperature Water Bath Antigen Retrieval

Jim Burchette, HT (ASCP)
Duke University Health System
Molecular Pathology
Immunopathology Laboratory
Durham, NC

burch007@mc.duke.edu

The world of immunohistochemistry (IHC) has drastically changed since the introduction of microwave antigen retrieval in 1991 by Shi, Key, and Kalra.[1] We have all used (and some still use) microwave ovens, autoclaves, vegetable steamers, hot plates, laboratory ovens, high temperature water baths, and newer immersion water bath modules to heat the various solutions and tissue sections for the purpose of exposing tissue antigens previously masked by aldehyde fixatives.

Today's practice of immunohistochemistry is deeply indebted to the pioneers of this now standard-of-care technology. Heat-induced epitope retrieval (HIER) technology has contributed to accurate diagnosis, and ultimately, better patient care. Problematic antibodies and antigens that were only conceptualized years ago are now routinely detected with ease. There are many articles published that address the history, offer overviews, and describe comparative studies of retrieval solutions and heat sources.[2-7] Rather than repeating this information, you are encouraged to review the references.

Materials and Methods

Duke Immunopathology Laboratory uses a batch approach for much of the off-line HIER, which requires high temperature water baths (Isotemp models 210 and 220) (Fisher Scientific, Waltham, MA) that achieve a maximum temperature of 100°C. The water bath technique is gentle and less disruptive to tissue sections, and the larger format accommodates more slide volume as compared to vegetable steamers and microwaves. We have implemented several ideas to make the HIER process more efficient.

To begin, a wire stain dish rack is placed in the high temperature water bath to hold plastic stain dishes, preventing them from tipping over (Fig. 1). These metal stain racks have been around for many years. A current source is MarketLab, Inc. (Caledonia, MI). Plastic Coplin jars with vinyl-coated lead wire wrapped around the bottom are used for smaller volumes of HIER solution. The lead wire provides enough weight to prevent the Coplin jar from tipping over. Coated lead wire can be obtained through scientific supply companies, including VWR (West Chester, PA). The use of lead wire and metal racks to anchor the solution vessels also allows the water level to be higher than the level of solution in the stain dish or Coplin jar. This should ensure even heat transfer.

The following retrieval solutions are used in the Immunopathology Laboratory.

Table 1. Solution Preparations

Target Retrieval Solution, pH 6.1*

This 10X concentrate is diluted 1:10 in distilled or deionized H_2O prior to use.

* Cat# S1699, Dako Corp.

0.1M Tris buffer, pH 9.5, 10X stock concentrate

Trizma HCl, FW 157.6	15.76 g
Trizma base, FW 121.1	12.11 g

Dissolve the above in 900 mL of distilled or deionized H_2O. Adjust to pH 9.5 and QS to 1000 mL.

10mM Tris buffer working solution

10X stock is diluted 1:10 in distilled or deionized H_2O prior to use.

0.1M EDTA buffer, pH 8.0, 10X stock concentrate

EDTA, FW 292.25	29.225

Pour EDTA powder into 800 mL of distilled or deionized water. Place the beaker on a stir plate.
Adjust pH to 8.0 using 4.0M sodium hydroxide. EDTA will go into solution as you approach pH 6.0-7.0.

10mM EDTA working solution

10X stock is diluted 1:10 in distilled or deionized H_2O prior to use.

0.1M Tris – 0.01M EDTA, pH 9.0, 10X stock

Tris Base	12.1 g
EDTA	3.7 g
Tween 20	5.0 mL

Add above reagents to 900 mL of distilled or deionized H_2O.

Adjust to pH 9.0

10mM Tris - 1mM EDTA working solution

10X stock is diluted 1:10 in distilled or deionized H_2O prior to use.

Table 2. Water Bath HIER Procedure

1. Place HIER solutions of choice in the high temperature water bath to preheat (Fig. 2).
2. Deparaffinize, clear, and hydrate slides to distilled or deionized H_2O using established histologic techniques.
3. Place slides in the preheated HIER solution. Monitor the solution temperature with a thermometer until the temperature reaches 98°C -100°C. Start the timer for the desired time. Most antigens require a 20 minute heating cycle; others require an extended heat cycle up to 40 minutes.
4. Remove slides and solution container from the water bath. Allow the slides to cool in the heated solution for 15 minutes.
5. Place slides and solution container in the water trough for 5 minutes (Fig. 3).
6. Gently wash the HIER solution from the slides with multiple changes of distilled or deionized H_2O.
7. Place slides in procedural buffer. Proceed with your IHC staining.

Fig. 2. Containers are placed inside the high temperature water bath to preheat the retrieval solutions prior to inserting the slides. Once the slides are placed, the lid is closed and the solution temperature is monitored with the dial thermometer. When the working temperature is reestablished, the timer is set for the desired length of the heating cycle. The water bath lid is open for demonstration purposes only and should remain closed throughout the procedure.

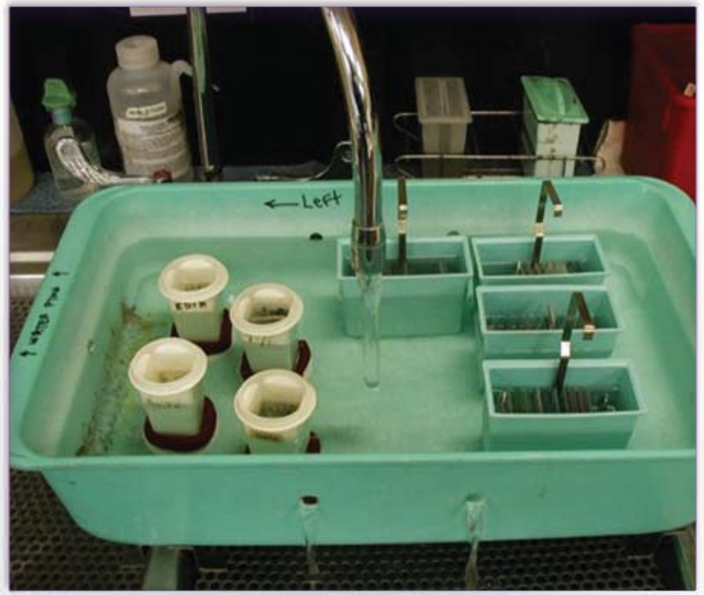

Fig. 3. Cool down water trough. Slides are cooled at room temperature for 15 minutes then placed in the water trough for 5 minutes. Holes are drilled slightly lower than the solution level in the stain dish to prevent the containers from tipping over.

Conclusion

In our hands, the high temperature water bath is the preferred device for heat-induced epitope retrieval. It provides for more uniform heating than other devices commonly employed for this purpose since the entire staining vessel can be submerged and surrounded by the high temperature water. As a result, our IHC staining results are consistently of high quality because fluctuations in temperature of the retrieval solution, as can occur when using a steamer or microwave, are eliminated.

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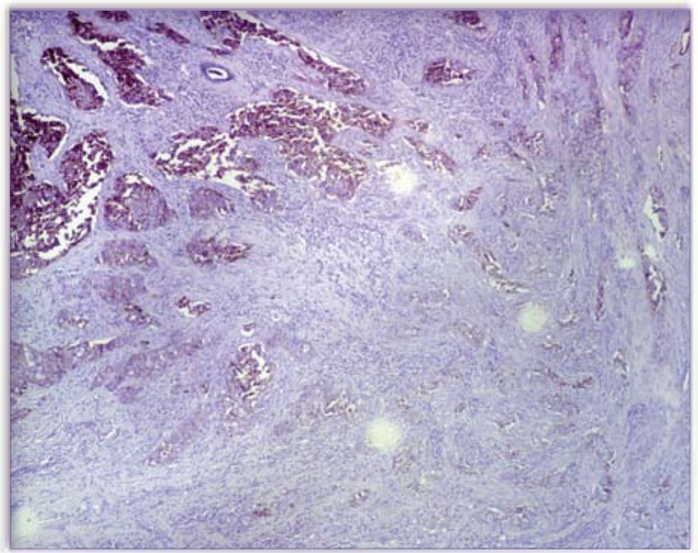

Fig. 1. IHC stain for HER2. Nests of cancer cells stain very intensely (dark brown color) near the periphery of what was a large sample (upper left corner). However, near the center of the specimen (bottom of image), similar nests of malignant cells stain faintly or not at all as a result of poor fixation. 40X

New Fixation Requirements for Breast Tissue Samples Will Alter Fixation Practices in Surgical Pathology Laboratories in 2008

Vinnie Della Speranza, MS, HTL(ASCP)HT
Medical University of South Carolina
Charleston, SC

dellav@musc.edu

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As evidenced by discussions on the listserv Histonet, histotechnologists and surgical pathologists are slowly becoming aware of the new fixation requirements being imposed onto CAP-accredited and CLIA-certified laboratories. These requirements take effect beginning in 2008 and affect all histopathology laboratories performing diagnostic workups and immunohistochemical staining on breast tissue samples.

A joint publication by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) appeared in the journal *Archives of Pathology and Laboratory Medicine* in January 2007. The ASCO/CAP publication was the result of an investigation by an expert panel convened by these organizations to conduct a systematic review of the scientific literature and to develop recommendations for optimal HER2 (human epidermal growth factor receptor 2) testing. This article represents the panel's findings, however, it should be understood that enforcement of the requirements set forth for HER2 testing laboratories delineated in the article is likely to occur during CAP accreditation or CLIA (Clinical Laboratory Improvement Act of 1988) inspections. This effort was largely prompted by increasing concerns that as much as 20% of current HER2 testing, both by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) techniques, may be inaccurate.[1]

The human epidermal growth factor receptor 2 gene, commonly referred to as HER2, is amplified in approximately 18%-20% of breast cancers. HER2 positivity is associated with worse prognosis (higher rate of recurrence and mortality) and appears to be associated with resistance to endocrine therapies (one common form of treatment for those who will benefit from it). It also seems to be predictive for either resistance or sensitivity to different types of chemotherapeutic agents.[1]

Of greatest importance, perhaps, are agents like trastuzumab (Herceptin®, Genentech, San Francisco, CA), a humanized monoclonal antibody that specifically targets and binds to the HER2 receptor found on the cell membrane of those patients who are positive for this marker; such agents have been found to be remarkably effective in a significant number of breast cancer patients. Data suggest that trastuzumab therapy improves treatment response rates, extends the disease-free interval and survival rates overall when used alone or in combination with chemotherapeutic agents in metastatic breast cancer.[1] As a result, it has been well established that identification of patients who would benefit from such therapy (eg, patients who are positive for the HER2 receptor) is an important component of breast cancer management. However, it must be noted that trastuzumab therapy is associated with serious potential side effects and is extremely costly. A 52-week regimen of trastuzumab costs approximately $100,000, according to the ASCO/CAP article. Therefore, the administration of trastuzumab to individuals who would not benefit poses significant disadvantages to those patients. For this reason, accurate determination of a patient's HER2 status is essential to identifying the patient population most likely to benefit from this therapeutic regimen. Testing should be performed on all patients with a diagnosis of metastatic breast cancer, however, the best method to assess HER2 status remains controversial. A more detailed discussion of this topic is beyond the scope of this article, however, the reader is encouraged to consult the large body of scientific literature on this topic.

Pick up any classical histology textbook and the reader will quickly find statements from authors emphasizing the importance of adequate (complete) fixation, which is intended, and indeed designed, to halt autolysis, microorganism-induced structural changes, and soluble molecule diffusion in tissues. So for the experienced histotechnology practitioner, the importance of prompt and adequate fixation of tissue samples is not only a given, but receives very strong emphasis in virtually every histology text as well as in the instruction of histotechnology students and neophyte technicians learning on the job.

Appreciation for the importance of adequate fixation is less apparent among residents and attending pathologists in clinical practice, as it is quite common in the clinical setting to find fixation times that can vary widely from one tissue sample to another in the very same laboratory. This may be affected by the time of day the surgery occurred or the time of day the sample arrived at the lab. In fairness, pathologists are under immense pressure from clinicians to provide very rapid diagnoses. Unfortunately, rushing samples through the pathology laboratory flies in the face of accepted fixation and processing standards that have been promoted in histotechnology texts for decades.

It bears emphasis that the requirement of "prompt" fixation is readily understood by most everyone in the discipline. Upon excision from the body, which eliminates sources of vital oxygen and nutrients to tissues, rapid immersion into the fixative of choice is necessary to limit the effects of autolysis and microorganisms. Fixation exerts its effects in part by stabilizing the structural proteins found in all bodily tissues, which allows a sample to display the expected morphologic appearance. Those who commonly practice immunohistochemical staining understand quite well the importance of preserving tissue antigens (typically proteins) if IHC staining is to be successful.

In the clinical setting, fixation of excised tissues is most often delayed due to events that occurred during the preanalytical phase of pathology testing. These include delays of immersion into fixative by the clinician collecting the sample, which is sometimes evidenced by a peripheral drying artifact in needle core biopsies, or delays in the delivery of fresh, unfixed tissues to the laboratory.

The concept of "adequate" or complete fixation seems less well understood among the surgical pathologists I have worked with. In practice, adequate or complete fixation is a function of several factors including the size (thickness) of the sample, the fixative used and its corresponding ability to penetrate tissues, and most importantly, the duration (time) the tissue is exposed to the fixative agent. What is most elusive for many is the notion that penetration of a fixative solution is *not* equivalent to fixation. This is especially true if one is using formaldehyde fixation, as is most commonly the case in the surgical pathology laboratory. The chemistry of formaldehyde fixation is well understood and has been described in exquisite detail in numerous texts and journal publications. However, the apparent change in the color of tissues exposed to formalin (a 10% solution of formaldehyde) often misleads individuals to believe that fixation has indeed been completed. Problems arise when tissues are insufficiently fixed in formalin because they often display the effects of alcohol fixation that occur during the typical tissue processing scheme, which utilizes alcohol dehydrants to remove water from tissues in preparing them to accept a supporting medium like paraffin.

Earlier work by Goldstein et al[2] explored how fixation may play a role in the discordance of results of IHC staining for estrogen receptor (ER) proteins between needle core breast biopsies and excisional samples from the same patient, witnessed in a percentage of cases at their facility. It is quite possible that this report and those of others have in part prompted or influenced the ASCO/CAP publication that has mandated the practice requirements for 2008.

Goldstein's group compared IHC staining for ER proteins among needle core and excised breast tissues after fixation in formalin for 3, 6, 8, 10, and 12 hours, as well as 1, 2, and 7 days. Both the intensity of staining and the percentage of positively stained cells were evaluated in each of the samples. In the same study, they also evaluated staining following heat-induced epitope retrieval (HIER) with an EDTA (ethylenediaminetetraacetic acid) buffer at pH 8.0 for 25 and 40 minutes.

The Goldstein group concluded that optimal ER IHC staining results are achievable when breast tissue samples are fixed in 10% formalin for a minimum of 6 hours. Interestingly, IHC staining dropped off after 10 hours of fixation if HIER techniques are only carried out for 25 minutes. When HIER is conducted for 40 minutes, IHC staining for ER appears to remain at the highest levels when breast tissue samples remain in formalin for at least 8 hours all the way up to 7 days. From the Goldstein paper, one can conclude that when utilizing heat-induced epitope retrieval for 40 minutes (as compared to 25 minutes), optimal IHC staining can be achieved when fixation is carried out for a minimum of 8 hours and up to a maximum of 7 days. It should be noted that the Goldstein group did not study the effects of fixation beyond 7 days.

A major deficiency of both the Goldstein and ASCO/CAP papers is that they fail to address tissue size or thickness, which relates directly to fixative penetration and fixation times.

While Goldstein notes that formalin, an aqueous fixative, penetrates tissues approximately 1 mm per hour, it is unclear that this generalization can be applied to all tissues, especially those heavily laden with lipid, such as breast tissues. In this author's opinion, until the grossing of tissues is sufficiently standardized in all surgical pathology laboratories to be able to attain consistent 3-4 mm slices of breast tissues, the effects of mandated fixation times are unlikely to remove all IHC staining variability, which appears to be the underlying intent of the ASCO/CAP paper.

The ASCO/CAP article mandates that breast tissues must be fixed for a minimum of 6 hours and no more than 48 hours. While the minimum fixation time is borne out by the Goldstein data, no explanation was offered for the imposed 48 hour maximum that will be enforced by CAP. This arbitrary maximum has been the source of considerable controversy as it poses significant operational difficulties for laboratories that are closed on weekends. A breast sample arriving on Friday morning may conceivably exceed the maximum allowable formalin exposure since many tissue processors sit in a delay mode until Sunday afternoon. Some have argued on various listservs that holding fixed breast tissues in alcohol to avoid overexposure to formalin violates the FDA requirements of the HER2 IHC testing kits available on the market. Most troubling, perhaps, is the failure of the ASCO/CAP panel to assess the relationship of epitope retrieval times to fixation times as the Goldstein group did, which makes the imposition of the arbitrary 48 hour maximum appear capricious since no data were offered to support or explain the rationale for the 48 hour maximum.

A number of other practice requirements are being imposed by CAP and are covered in greater detail in the ASCO/CAP article. The reader is encouraged to read that document carefully to better understand the adjustments in procedure that may be necessary to institute in 2008.

References

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2. Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F. Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. *Am J Clin Pathol.* 2003;120(1):86-92.

Mark Your Calendar! Educational Opportunities in 2008

JANUARY

18 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Prostate Cancers Aren't All Alike. What Tools Can Help Us Sort Them Out?**
Speaker: Dean Troyer, MD
Professor of Pathology and Urology
University of Texas Health Science Center
San Antonio, TX

23 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Substitutes: Formalin, Alcohol and Xylene**
Speakers: Ada Feldman, MS, HTL(ASCP)HT
Dee Wolfe, HT(ASCP)
Anatech Ltd
Battle Creek, MI

FEBRUARY

15 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Microwave Use in the Laboratory**
Speaker: Donna Willis, HT/HTL (ASCP)
Milestone Medical
Grand Prairie, TX

27 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Histochemical and Immunocytochemical Stains: Their Use for Cytologic Specimens**
Speaker: Donna K. Russell, MS, CT(ASCP)HT
University of Rochester Medical Center
Rochester, NY

MARCH

7-8 **New York State Histotechnological Society**
Site: Holiday Inn
Saratoga Springs, NY
Contact: Mary Georger
Email: Mary.Georger@rochester.edu

8 **Arkansas Society of Histotechnologists Spring Meeting**
Contact: Shane Jones
Email: shane.jones@baptist-health.org

14-15 **Kentucky Society for Histotechnology**
Site: Galt House
Louisville, KY
Contact: Mary Beth Knight
Email: marybeth.knight@nortonhealthcare.org

21 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Dermatopathology: A Guide for the Histologist**
Speaker: Clifford Chapman, MS, HTL(ASCP)QIHC
Children's Boston Hospital
Boston, MA

MARCH (CONT.)

26 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Coding for Histotechs**
Speaker: Bonnie Whitaker, MT(HEW), HT(ASCP)QIHC
Brown and Associates
Houston, TX

29 **Nevada Society of Histotechnology**
Site: Sunrise Hospital Medical Center
Las Vegas, NV
Contact: Connie Grubaugh
Email: conniegrubaugh@hotmail.com
Phone: (702) 396-4079

APRIL

3-5 **Region III meeting hosted by Georgia Society of Histotechnology**
Site: Westin Peachtree Plaza
Atlanta, GA
Contact: Mike Ayers
Email: mike.ayers@piedmontnewnan.org

11-12 **Colorado Society of Histotechnology**
Site: Millennium Harvest House
Boulder, CO
Contact: Stacey Langenberg
Email: Stacey.langenberg@comcast.net

17-19 **North Carolina Society for Histopathology Technologists Spring Meeting**
Site: Sheraton New Bern Hotel & Marina
New Bern, NC
Contact: Wanda Jones
Email: wanpto@aol.com

18 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Histological Techniques and Special Stains for Undecalcified Bone**
Speaker: Nancy Troiano, MS
Yale University School of Medicine
New Haven, CT

18-19 **Histology Society of Ohio State Convention**
Site: Holiday Inn
Independence, OH
Contact: Amy Aulthouse
Email: a-aulthouse@onu.edu

19-21 **Texas Society for Histotechnology**
Site: Hilton DFW Lakes
Grapevine, TX
Contact: Kathy Dwyer
Email: Kathleen.a.dwyer@questdiagnostics.com

23 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Role of Immunohistochemistry in Prostatic Lesions**
Speaker: Mitual Amin, MD
William Beaumont Hospital
Royal Oak, MI

23-25 **Tri-State Histology Conference (MN, IA & WI)**
Site: Hotel Sofitel
Bloomington, MN
Contact: Lois Rowe rowe.lois@mayo.edu
Colleen Forster cforster@umn.edu

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MAY

15-18 **California Society for Histotechnology**
Site: Marriott Ventura Beach Hotel
Ventura, CA
Contact: Robin Simpkins
Email: rsimpkins@memorialcare.org

16 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **An Advanced Look at Immunohistochemistry Mathematics in the Laboratory**
Speaker: Joel Martinez, BS
BIOCARE Medical
Houston, TX

16-17 **Michigan Society of Histotechnologists**
Site: Thomas Edison Inn
Port Huron, MI
Contact: Paula Bober
Email: boberp@michiganurology.com
Phone: (734) 207-5235

16-18 **Florida Society for Histotechnology**
Site: Bahia Mar Beach & Yacht Resort
Fort Lauderdale, FL
Contact: Jerry Santiago (904) 244-6149
Sue Clark (954) 987-2000 x 5371
Email: fsh@fshgroup.org

28 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Solvent Recycling – Anyone Can Do It!**
Speaker: Kim Szczepanek, HT(ASCP)
CGB Biotech, Ltd.
Columbus, OH

JUNE

20 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Artefacts of Microtomy, Staining and Coverslipping: Causes & Corrections**
Speaker: Denise Long-Woodward, MS, HTL(ASCP)QIHC
University of Connecticut, Dept. of Pathobiology and Veterinary Sciences
Mansfield, CT

20-21 **Tennessee Society for Histotechnology Annual Meeting**
Site: Valley View Lodge Convention Center
Townsend, TN
Contact: Jerry Meade
Phone: (865) 995-2064 or (865) 742-4460
Email: jmeade0710@aol.com

25 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Her2/neu Testing for Breast Cancer: Standardization and Validation**
Speaker: Elizabeth Sheppard, MBA, HT(ASCP)
Ventana Medical Systems
Tucson, AZ

JULY

18 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Immunohistochemistry Markers, Traditional and New**
Speaker: Traci DeGeer, BS, HTL(ASCP)QIHC
Ventana Medical Systems, Inc.
Tucson, AZ

23 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Frozen Sectioning**
Speaker: Carole Barone, HT(ASCP)
A. I. Dupont Institute
Wilmington, DE

AUGUST

15 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Taking the Complexity Out of Validation, Verification and Controls**
Speaker: Elizabeth A. Sheppard, MBA, HT(ASCP)
Ventana Medical Systems, Inc.
Tucson, AZ

27 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 0535-4060
histo@nsh.org
Title: **Bacterial Staining Methods**
Speaker: Denise Long-Woodward, MS, HTL(ASCP)QIHC
University of Connecticut, Dept. of Pathobiology and Veterinary Sciences
Mansfield, CT

SEPTEMBER

12-18 **NATIONAL SOCIETY FOR HISTOTECHNOLOGY**
Annual Symposium/Convention
Pittsburgh, Pennsylvania
Contact: Aubrey Wanner, NSH Office
Phone: (443) 535-4060
Fax: (443) 535-4055
Email: Aubrey@nsh.org

19 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Sjogren's Syndrome, Clinicopathologic and Diagnostic Aspects**
Speaker: H. Stanley McGuff, DDS
Professor, Pathology Department
University of Texas Health Science Center
San Antonio, TX

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OCTOBER

17 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **10 Safety Mistakes in the Histology Laboratory and How to Avoid Them**
Speaker: Jason Burrill, BA, HT(ASCP) SLS
Charles River Laboratories
Wilmington, MA

22 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Carbohydrate Stains**
Speaker: Caridad Gutierrez, MEd, HTL(ASCP)
Miami Dade College
Miami, FL

NOVEMBER

19 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Use of Radiofrequency Energy in Surgery and Its Effects on Staining**
Speaker: Janet Maass, ME, HT(ASCP)HTL, CT(ASCP)
Covidien
Boulder, CO

21 **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **Changes to the CAP Anatomic Pathology Checklist Requirements and/or Common Deficiencies Identified in AP Labs in the Course of CAP Inspections**
Speaker: Francis E. Sharkey, MD
Director, Surgical Pathology
University of Texas Health Science Center
San Antonio, TX

DECEMBER

17 **NSH Teleconference 1:00 pm Eastern Time**
Contact: (443) 535-4060
histo@nsh.org
Title: **Special Stains for the Kidney Biopsy**
Speaker: Maryann A. Farinola, MD
William Beaumont Hospital
Royal Oak, MI

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