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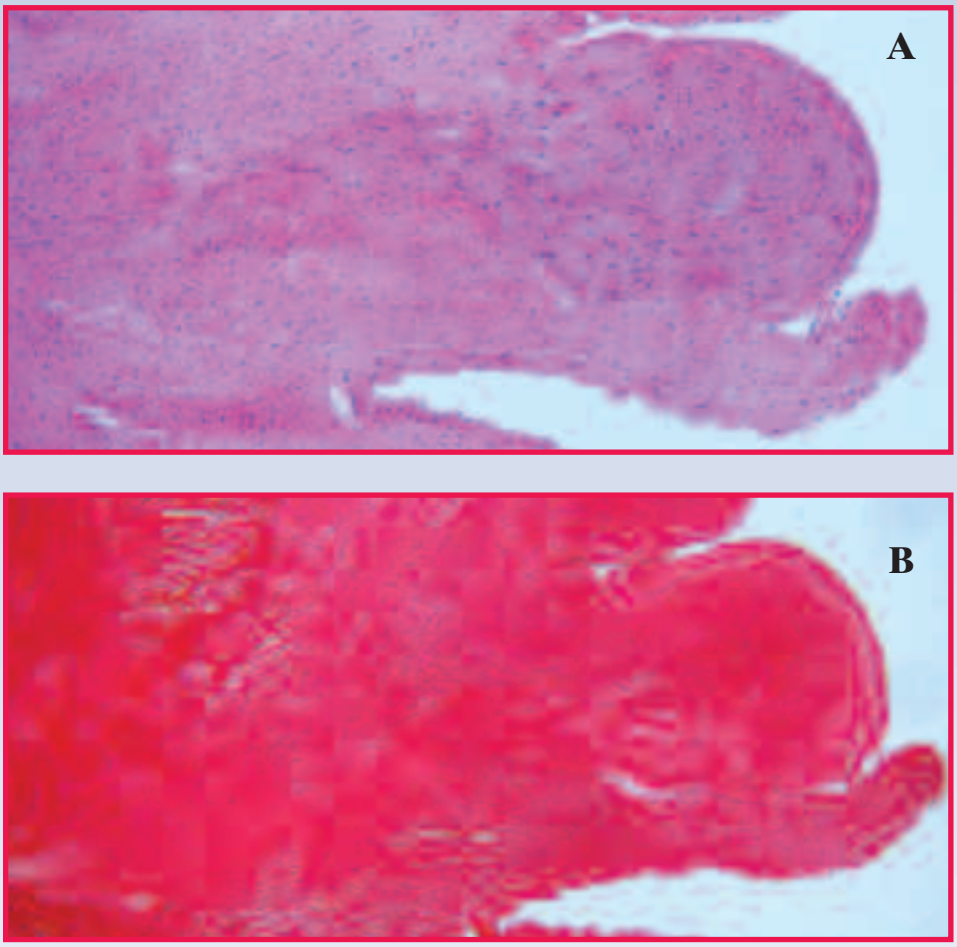

Fig. 1. Heat effect can be seen with H&E, picro Sirius red, and spectral imaging. A) Porcine artery, 40X, H&E stain, illustrates cytoplasmic staining with a bluish cast in heat-affected areas. Nuclei appear disrupted or absent. B) Porcine artery, picro Sirius red stain, 40X, with light microscopy is not informative unless viewed with spectral imaging. C) Porcine artery, picro Sirius red stain, 40X, viewed with spectral imaging. Red areas represent the most heat-affected portions of the sample, while the blue areas are moderately affected. Green areas are not affected. Note that heat effect has traveled into the normal tissue area.

The Effect of Radiofrequency Energy in Tissue

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Abstract

Monopolar and bipolar radiofrequency (RF) energy applied to tissues during surgery may cause histological stains to act differently than they would on unaffected tissue. Heat from the RF device not only affects the edge of the tissue, but it may also propagate from the original application site into the tissue and affect adjacent cellular structures.[1]

Introduction

New technologies are now being used for surgical hemostasis, so it is important for histology practitioners to be aware that RF energy can alter the colors of staining results, as well as cause some artifacts in

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tissue. The RF energy that is used during electrosurgery, however, should not be confused with the current microwave technologies that apply microwave energy to control heat generated during tissue sample processing.

Laparoscopic surgeries are typically done through two or three small incisions rather than one large, open incision. One small incision may be used for a light and camera while another incision might accommodate a surgical device inserted through a tube that is holding the incision open. This less invasive surgical technique results in faster recovery time, which can mean that no overnight hospital stay is required. Oftentimes, RF energy is used rather than sutures or staples to seal bleeders, isolated vessels, and tissue bundles, which helps to expedite surgery. When tissue is removed during surgery, it is brought out through the tube holding the incision open. Due to the surgical procedure, this tissue might include RF-affected portions. RF electrosurgery is also commonly used during open surgical procedures to reduce blood loss during surgery and limit operating room time.[2]

The effect of RF is typically noted by the cauterizing effect on the tissue edge, but if one looks closely, the heat effect may have propagated some distance from the edge (Fig. 1). Our bodies have a unique way of handling heat-affected tissues through the healing process. Sealed tissues, which are a product of cauterization, ideally do not contain any foreign materials, allowing our bodies to heal faster as a result.

Water vaporization can occur in the tissues during RF electrosurgery, and may be identified as a sectioning artifact. However, this may be heated water vapor, which starts superficially and then goes deeper into the tissue. Vaporization (popcorn effect) looks like small holes in the tissues, and is often found adjacent to the heated area (Fig. 2). The cauterized tissues may be challenging or difficult to section depending upon what type of RF energy was used and to what extent the energy was used in surgery. The clue to good sectioning includes using a high-profile blade in the microtome, soaking the block, and sectioning the tissue longitudinally rather than cross-sectioning to the heat-affected line.

Radiofrequency Energy

Unlike the standard electrical currents that we use to power our homes, which alternate at a frequency of 60 Hz (cycles per second), electrosurgical systems function at radio frequencies, which are above 100 kHz, in order to prevent nerve and muscle

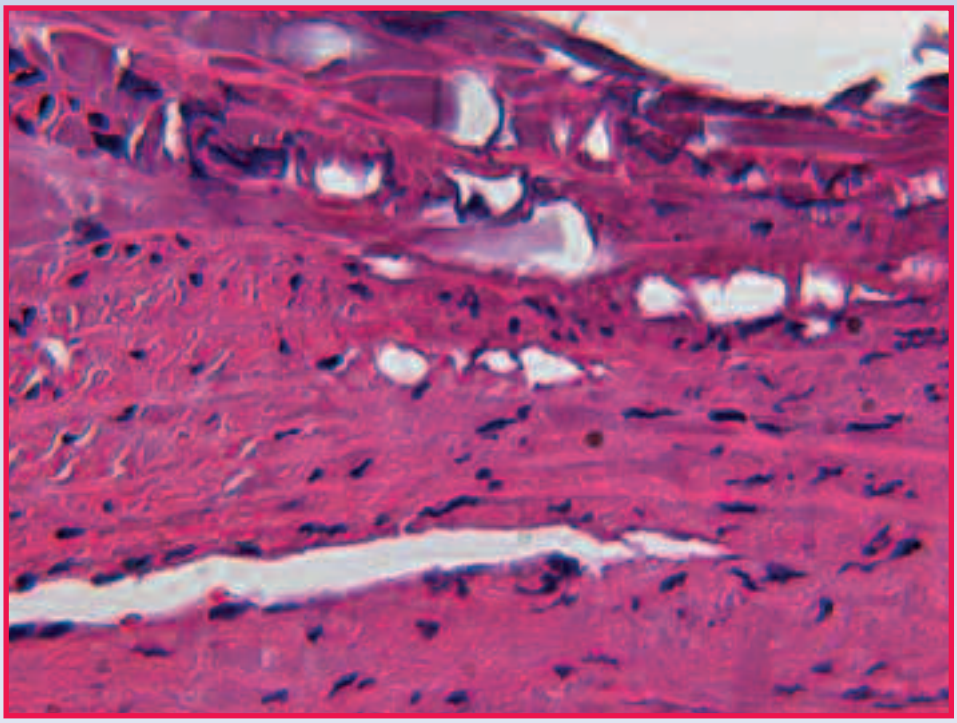

Fig. 2. Water vaporization (popcorn effect) with holes in the tissue. H&E, 40X

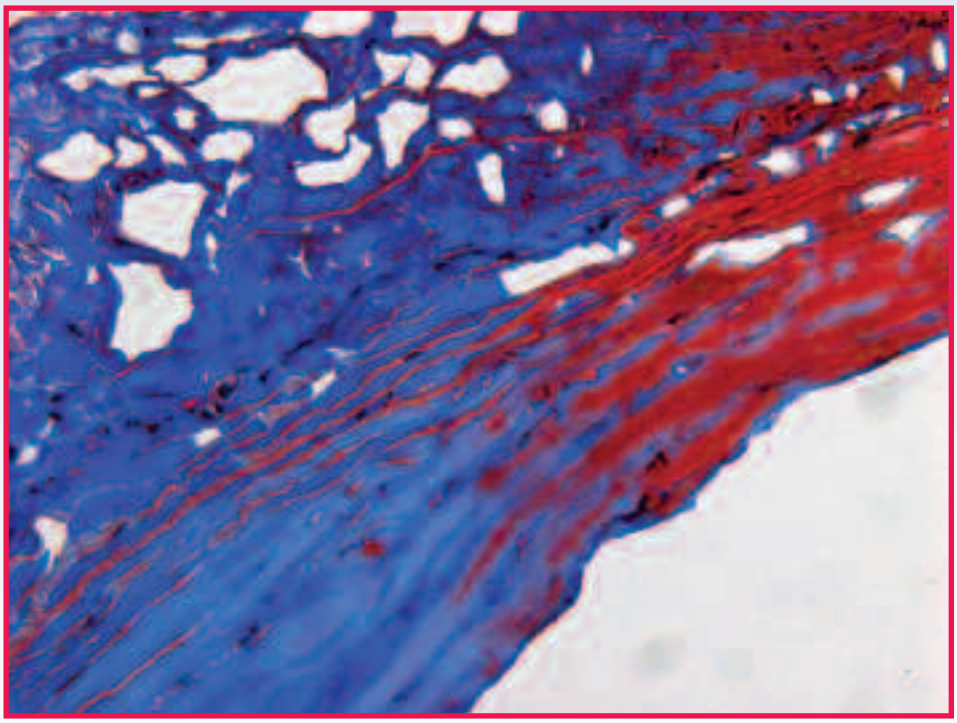

Fig. 3. In the Masson trichrome stain where the RF energy was intense, the collagen appears red rather than blue. Also, the heat-affected collagen appears a darker blue while the unaffected collagen appears a bright blue. Masson trichrome, 40X

stimulation and, ultimately, electrocution. These higher frequencies used in electrosurgical energy can pass through a patient with negligible neuromuscular stimulation and no risk of electrocution. In all RF electrosurgery, there is an active and a return electrode, so that electric waveforms at various amplitudes can pass between the two to achieve the desired tissue effect.[2]

Monopolar electrosurgery is very common due to its convenience and clinical effectiveness. In this type of electrosurgery, the active electrode is the surgical site where an electrosurgical pencil is used to cut or coagulate tissues, and the return electrode is a pad placed somewhere else on the patient's body. Tissue desiccation occurs when the active electrode comes in contact with the tissue. By altering the power setting, the surgeon can create a range of minimal to extensive thermal spread depending upon the desired effect.[2]

In bipolar electrosurgery, both the active and return electrodes are contained at the surgical site where the electricity passes between two opposing electrodes. Only the tissue grasped between the electrodes is included in the electrical circuit, so no patient return electrode is needed. The effects achieved by bipolar electrosurgery, while more contained than in monopolar electrosurgery, are unique due to the differences in how the two systems work. The LigaSure™ (Valleylab, Boulder, CO) vessel sealing system is a bipolar electrosurgical system that applies a feedback-controlled amount of energy in combination with mechanical pressure to fuse vessel walls and create a permanent seal for tissue bundles and isolated vessels up to and including 7 mm. This eliminates the need for clips or sutures, virtually ensuring that no foreign materials are left behind in the body. Because these bipolar seals are made under pressure, the resulting highly desiccated tissue can lead to difficulty in histological processing of the samples and ultimately in interpreting the differences between affected and unaffected tissues.

Methods

RF energy (monopolar and bipolar applications) was applied to fresh necropsied porcine and ovine tissues. Tissues were fixed in 10% formalin for 24 hours and processed on a tissue processor (using no heat, except for paraffin) on varying cycles, depending on the size of the tissue. Tissues were embedded in hard paraffin and sectioned at 4 microns.

Heat from different kinds of surgical instruments affects tissues in unique ways. When instruments are developed, one of the considerations is to minimize any heat affect. To identify heat affect in tissue, one looks at tissue components such as collagen, elastic fibers, endothelium, reticular fibers, etc. In this study, the following staining methods were used to demonstrate artifacts seen in heat-affected tissue: hematoxylin and eosin, picro Sirius red,[3] spectral analysis[4] (Fig. 1), Masson trichrome[5] (Fig. 3), Gordon and Sweet's reticulin[6] (Fig. 4), heat shock protein 70 (Fig. 5), von Willebrand (Fig. 6), and elastic stain (Fig. 7).

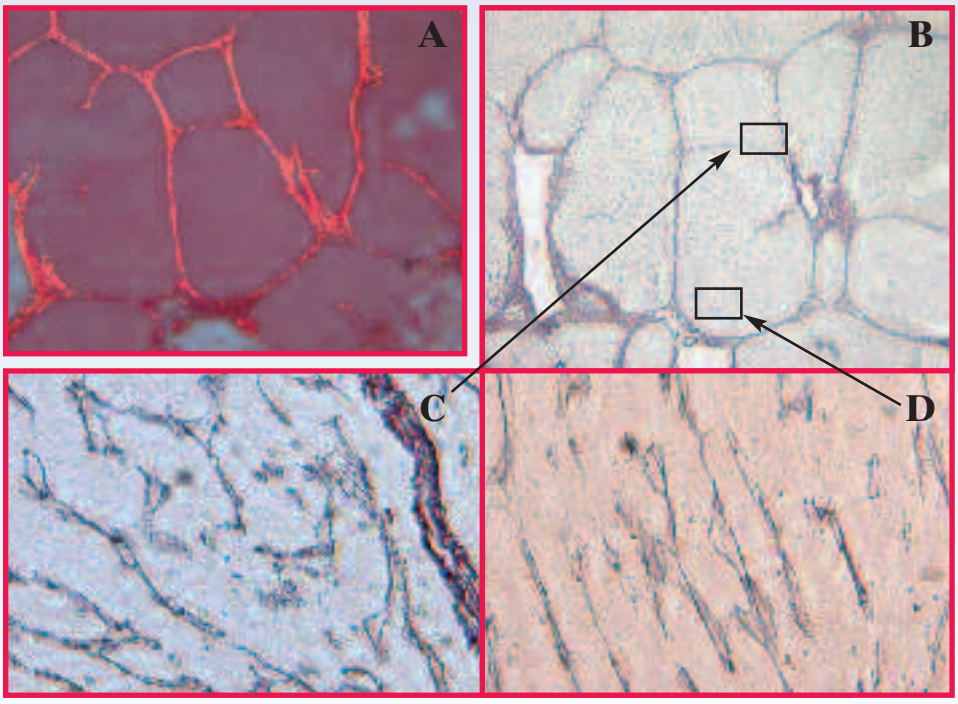

Fig. 4. All images are from the same section of porcine liver where heat has been applied to the edge. A) Viewed with picro Sirius red using polarization, note that the collagen is not damaged far into the tissue. 20X; B) Using Gordon and Sweet's reticulin stain, one can see rearrangement of reticular fibers near the heat-affected edge. 20X; C) This is from an area in the upper lobule seen in B that exhibits normal reticular fibers. Gordon and Sweet's, 200X; D) This is from a portion of the lower lobule seen in B that exhibits elongated reticular fibers that resulted from their proximity to the heat source. Gordon and Sweet's, 200X

Results

Tissue desiccation from RF energy tends to bring about cell and tissue shrinkage, making the tissue appear more dense than hydrated tissues. Thus, tissues appear to be more intensely stained. In Fig. 1, the hematoxylin and eosin-stained porcine artery demonstrates an eosinophilic or blue cast in the heat-affected area. In the picro Sirius red slide, the dark red color is in the same area, and when one applies spectral imaging, the heat lines are better identified. Spectral imaging is the process of overlaying a false color, which one links to a chosen true color in the image, by allowing the computer (which can identify colors better than the human eye) to match pixels with that same color and overlay the false color.

The Masson trichrome stain is used to identify collagen. As histotechnologists, we generally identify collagen as blue and muscle as red. When tissue is desiccated from RF energy oftentimes the collagen is a darker blue than usual, but it can also stain red (Fig. 3). Collagen can also be identified in tissue by staining with picro Sirius

red and polarizing the tissue. When a Gordon and Sweet's reticulin is done on the same section in the porcine liver lobule, it shows reticular fibers that have been rearranged in the lobules in which RF energy has been applied to the edge (Fig. 4). When immunohistochemical stains are performed on tissues that have been affected by RF energy, one may see unusual staining patterns. An example is endothelium that does not stain; yet in the same vessel, proper staining takes place elsewhere. The same thing may happen with heat shock protein 70 (Figs. 5 and 6). Another tissue component that has demonstrated energy effect is the elastic fiber. Sometimes the area will not differentiate properly or the fibers may not hold the stain, but they can be seen under high power. (Fig. 7).

Conclusion

Using RF energy during surgery causes localized tissue damage, yet it may result in better healing because it minimizes the possibility of foreign material being left behind in the body. Ultimately, it is the surgeon who can best decide the optimal method to use in each situation during surgery, but histology technicians and technologists need to be aware that tissues they are processing might have been altered during surgery with RF electrosurgical techniques. A closer look at the tissue condition rather than just the staining result will allow a more accurate assessment when we think a stain has failed and needs to be redone.

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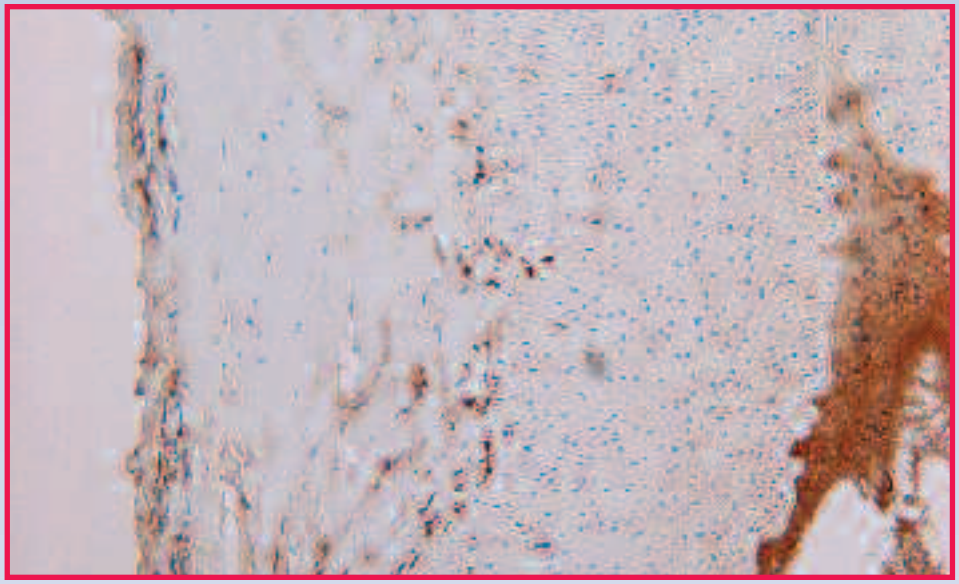

Fig. 5. Abnormal staining on the edge where the intense RF energy was applied, compared to more normal staining on the opposite side of the energy zone. Peroxidase Dako EnVision+ 1:1200 heat shock protein 70, 20X

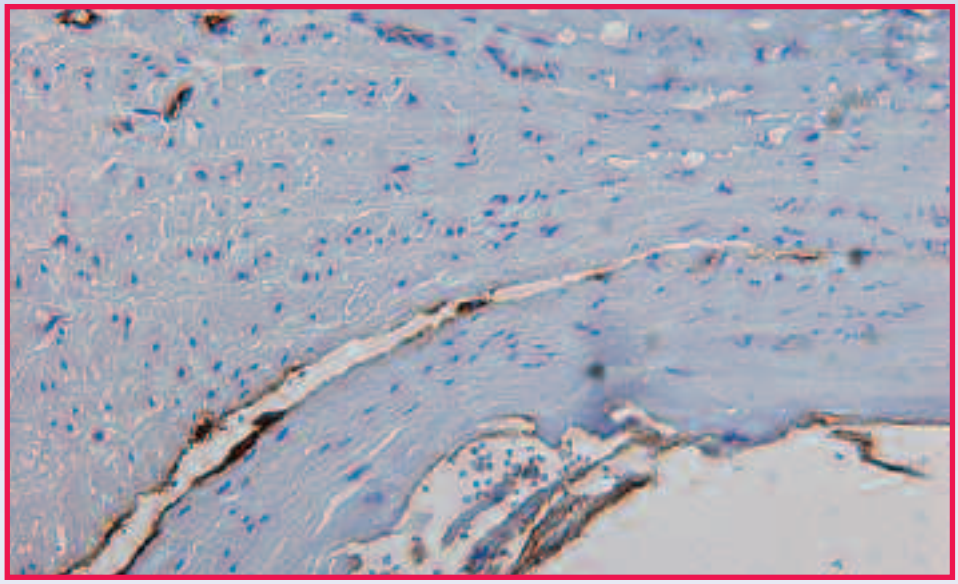

Fig. 6. One can see in the heat-applied area the endothelium lacks staining, but away from the heat-affected area, the endothelium stains normal. Peroxidase Dako LSAB+ 1:4000 von Willebrand (factor VIII), 20X

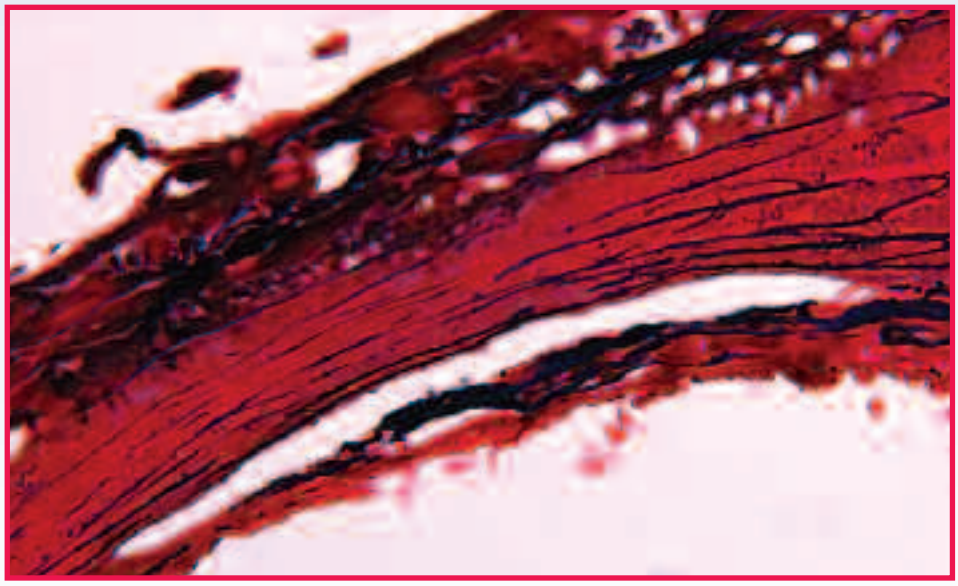

Fig. 7. The elastic fibers can be well defined, unstained, or there can be tissues, which cannot be differentiated to see the fibers, counterstained with picro Sirius red. Picro Sirius red, 100X

Microwave Histoprocessing: One-step Dehydration and Clearing of Articular Cartilage and Synovial Tissue

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Introduction

Routine histoprocessing procedures for articular cartilage and synovial tissue remain laborious and time-consuming. Fortunately, recent availability of laboratory microwave technology offers new opportunities to safely shorten processing times and improve reproducibility without compromising results.[1,2] Other advancements, including microwave-specific reagents such as the one-step dehydration/clearing agent, JFC solution (Milestone, Italy), further facilitate microwave processing. JFC solution, which is a mixture of absolute ethanol and isopropyl alcohol with long-chain hydrocarbons, simplifies processing. There are essentially two steps: JFC and paraffin. This streamlined approach can reduce processing times to between 0.5 to 2.5 hours, depending on the size and nature of tissue specimens. The applicability of new microwave methods to various tissue types and for differing histological techniques remains to be fully studied, especially in the area of orthopedic research. In this study, femoral articular cartilage and patellar synovial tissue samples from rabbits were processed using newly developed microwave protocols, and the histological quality and reproducibility were compared to tissues prepared using traditional manual histoprocessing.

Materials and Methods

Tissue samples were obtained fresh from animals necropsied for other studies. Eight rabbit femoral, full-thickness articular cartilage samples and 8 patellar synovial tissue samples were fixed in 10% neutral buffered formalin (NBF) at room temperature for 72 hours, in addition to 2 full-thickness articular cartilage samples fixed in 4% paraformaldehyde for immunohistochemical studies. Prior to histoprocessing, all articular cartilage was decalcified in 0.5M HCl/0.1% glutaraldehyde for appropriate times. Following decalcification, tissue samples were then trimmed to equal dimensions of 1.0 x 0.9 x 0.7 cm for articular cartilage, and 0.8 x 0.5 x 0.2 cm for synovial tissue. Finally, half of all samples were processed using a laboratory microwave (MicroMed T/T Mega, Milestone Inc, Italy) and JFC solution, while remaining samples underwent traditional manual procedures. Table 1 describes the optimized microwave process specific for cartilage and synovium. Microwaves featuring automatic control allow for simple programming of multiple steps. Table 2 shows the overall scheme of the procedures.

After histoprocessing, all paraffin-embedded samples were cut into 5-micron thick sections with a microtome (Leica-2155) (Leica MicroSystems, Bannockburn, IL), and observations of cutting ease and block characteristics were recorded. Articular cartilage sections were stained using a previously described safranin O/fast green method[3] (0.1% safranin O, 0.01% fast green) for glycosaminoglycan content. Other sections were immunostained with collagen type II antibody (II-II6B3, Developmental

Table 1. Detailed microwave procedures for articular cartilage and synovial tissue

Microwave Procedures			
DEHYDRATION STEP			
Step	**Time**	**Power**	**Temperature**
1	00:20:00	400W	40°C
2	00:03:00	200W	45°C
3	00:20:00	300W	50°C
4	00:03:00	200W	55°C
5	00:20:00	350W	65°C
6	01:15:00	250W	70°C
WAX IMPREGNATION STEP			
Step	**Time**	**Power**	**Temperature**
1	00:20:00	600W	79°C
2	00:05:00	300W	79°C
3	00:20:00	600W	83°C
4	00:05:00	300W	83°C
5	00:20:00	500W	85°C
6	00:30:00	300W	85°C

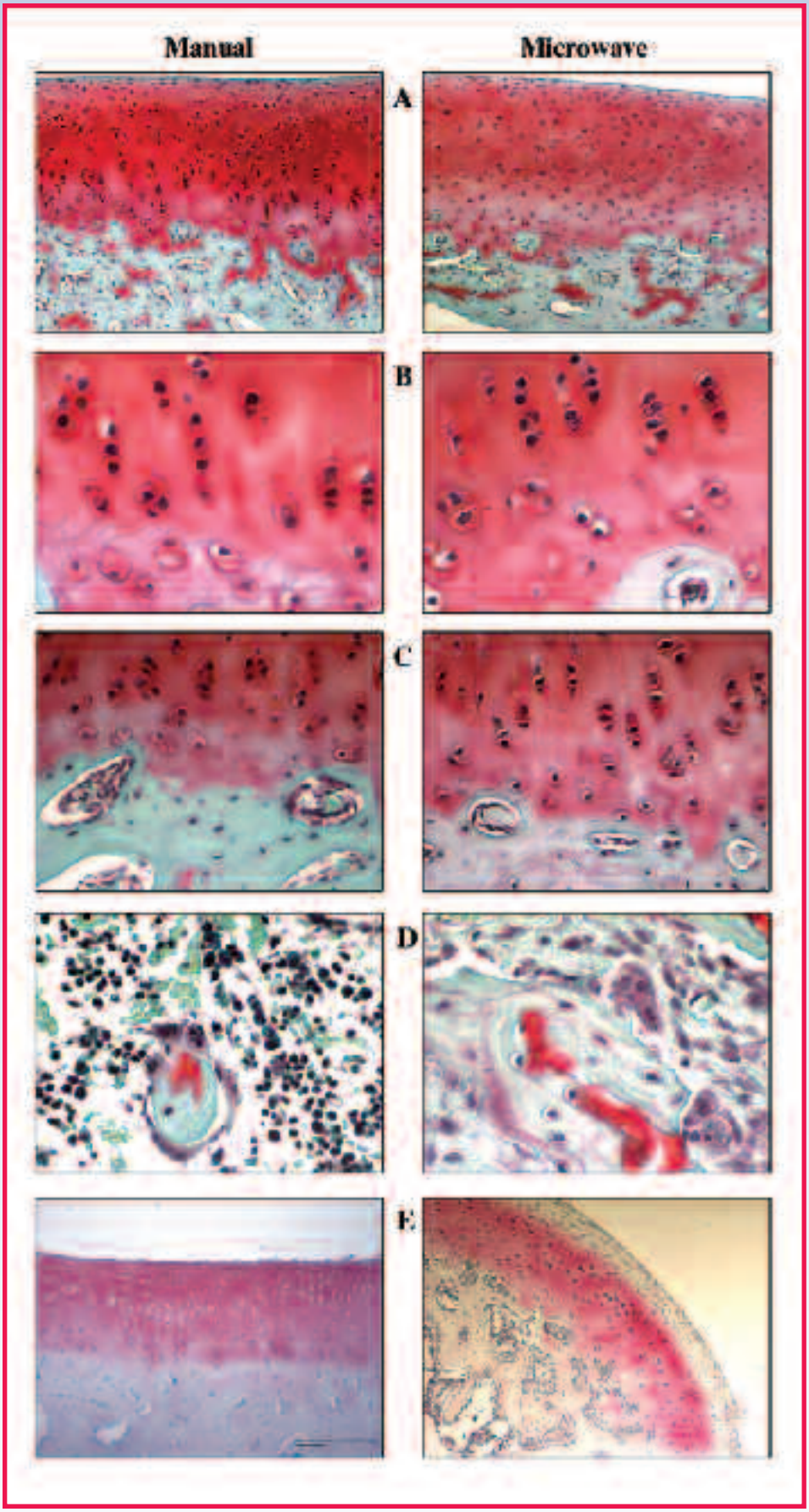

Fig. 1. Histological and immunohistochemical comparison of manual and microwave histoprocessing: A) Rabbit articular cartilage. Safranin O, 10X; B) Rabbit articular cartilage glycosaminoglycan in proximity of columnar chondrocytes. Safranin O, 40X; C) Rabbit subchondral bone. Safranin O, 40X; D) Rabbit osteoclasts in proximity to growth plate. Safranin O, 40X; E) Rabbit articular cartilage immunostained for collagen type II. 10X

Studies Hybridoma Bank, University of Iowa), using a 1:10 primary antibody dilution in 10% goat serum/PBS and a 1 hour incubation at room temperature. Visualization was achieved with the Vectastain ABC-AP System (AK-5000, Vector Laboratories Inc, Burlington, Ontario, Canada). All synovial tissue sections were cut to a 5-micron thickness and stained with hematoxylin-eosin.

Stained microwave slides were compared histologically to the manually processed slides on the basis of specimen morphology, prevalence of artifacts, and specific cartilage and synovium characteristics.

Results

The development of standardized processing protocols using laboratory microwave technology represents a viable alternative for improving efficiency and reproducibility in histology laboratories. In this study, microwave histoprocessing of rabbit articular cartilage and synovial tissue using one-step dehydration and clearing with JFC solution was optimized. Microwave processing significantly shortened overall processing times from 46 hours to 3¼ hours for cartilage samples, and from 8 hours to 3¼ hours for synovium (Table 2). Additionally, use of the microwave and JFC precluded the need for xylene and toluene, thus contributing to a safer working environment. During sectioning, it was observed that all paraffin blocks of both cartilage and synovium were neither hard nor brittle, and were cut well. Interestingly, it appears that some residual hydrocarbon chains from the JFC solution remained in the embedding paraffin, since blocks prepared via microwave were noticeably easy to cut.

Figure 1 demonstrates the high quality of the prepared specimens following both microwave and

manual processing. There was minimal section shrinkage, and a distinct lack of alteration in cell morphology or swelling of connective tissue fibers was noted. In addition, these unique orthopedic tissues had easily identifiable cartilage layers and different cell types, including chondrocytes (Figs. 1A, 1B, 1C) and osteoclasts in close proximity to the growth plate (Fig. 1D). In cartilage, glycosaminoglycan quantity and distribution was effectively observed following safranin O staining, regardless of the histoprocessing method (Fig. 1B), and despite the seemingly high temperature (70°-85°C) and energy ranges (300-600 W) required for microwave processing. Likewise, the identification of collagen type II in articular cartilage through immunohistochemical means was similarly feasible for both methods (Fig. 1E). The high quality of synovial tissue specimens following microwave histoprocessing (Figs. 2A, 2B) was such that it permitted a quantitative histological study of inflammation and cellular alterations in rabbit synovial tissue during the course of another study.

Conclusion

Microwave histoprocessing consistently gave high-quality, reproducible, and equivalent histological results when compared with routine manual histoprocessing for both rabbit articular cartilage and synovial tissue. Microwave use, however, brings the significant benefit of much shorter histoprocessing times in a safer environment, and should be considered in clinical, academic, and industrial settings.

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Table 2. Procedural summaries for articular cartilage and synovial tissue processing

Processing Procedures			
ARTICULAR CARTILAGE			
Manual		**Microwave**	
Step	**Time**	**Step**	**Time**
1. Rinsing		Rinsing	
PBS	45 min	PBS	45 min
2. Dehydration		One-step dehydration/clearing	2 hr
70% Ethanol	overnight (>15 hr)		
95% Ethanol	4 hr		
100% Ethanol	4 hr 30 min		
3. Clearing			
Cedar wood oil	overnight (>15 hr)		
Toluene	20 min		
4. Paraffin impregnation	6 hr at 62°C	Paraffin impregnation	30 min
Total time	**~46 hr**	**Total time**	**~3 hr 15 min**
SYNOVIAL TISSUE			
Manual		**Microwave**	
Step	**Time**	**Step**	**Time**
1. Rinsing		Rinsing	
PBS	40 min	PBS	45 min
2. Dehydration		One-step dehydration/clearing	2 hr
70% Ethanol	30 min		
80% Ethanol	30 min		
95% Ethanol	60 min		
100% Ethanol	80 min		
3. Clearing			
Xylene	60 min		
Toluene	20 min		
4. Paraffin impregnation	3 hr at 62°C	Paraffin impregnation	30 min
Total time	**~8 hr**	**Total time**	**~3 hr 15 min**

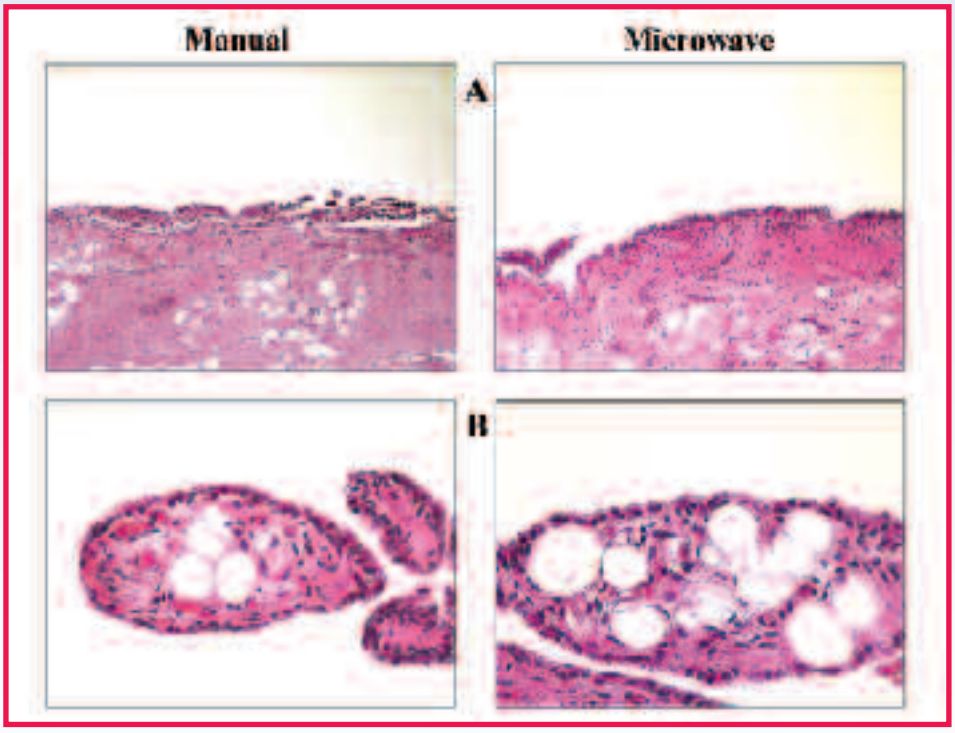

Fig. 2. Histological comparison of manual and microwave histoprocessing of synovial tissue: A) Rabbit synovial tissue. H&E, 10X; B) Rabbit synovial tissue. H&E, 40X

Comparison of Rabbit Monoclonal Antibodies to Their Mouse Monoclonal Analogs

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Abstract
It has been known for many years that the rabbit polyclonal antibodies are more sensitive than mouse antibodies for various antigens in tissue sections, but often show more nonspecific labeling. Conversely, monoclonal antibodies are known to be more specific than polyclonal antibodies, but often lack sensitivity. Rabbit monoclonal antibodies (RabMAbs) have been shown to have greater sensitivity than mouse monoclonal antibodies (MoMAbs) for many antigens, and are as specific as mouse monoclonals. In this study we compared the immunohistochemistry of rabbit monoclonals to their mouse monoclonal analogs. Rabbit monoclonals showed real increases in sensitivity and none of the nonspecific labeling common to polyclonals.

Rabbit Monoclonal *Mouse Monoclonal*

Estrogen Receptor

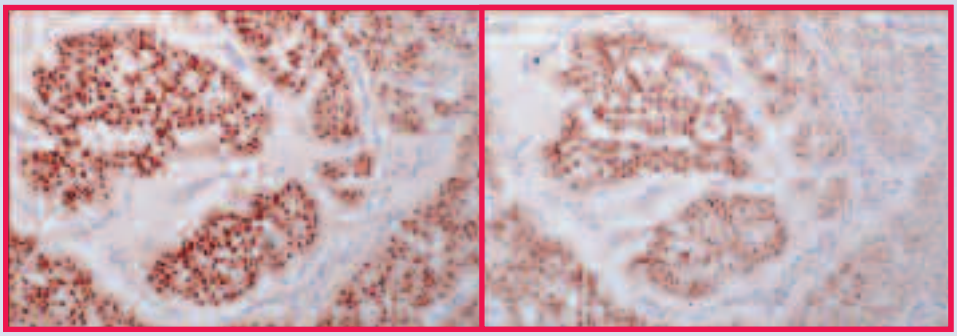

Fig. 1A. Clone SP1. Breast carcinoma, 200X

Fig. 1B. Clone 6F11. Breast carcinoma, 200X

Progesterone Receptor

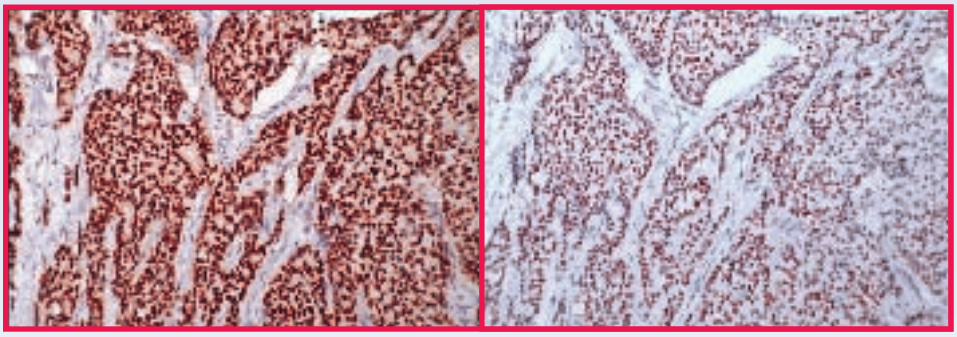

Fig. 2A. Clone SP2. Breast carcinoma, 200X

Fig. 2B. Clone 1A6. Breast carcinoma, 200X

C-erbB-2

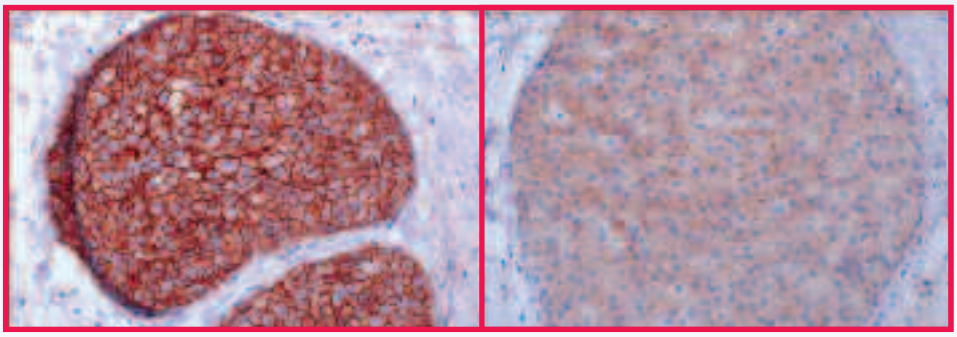

Fig. 3A. Clone SP3. Breast carcinoma, 200X

Fig. 3B. Clone CB11. Breast carcinoma, 200X

Introduction

Rabbit monoclonal antibodies are a recent development for diagnostic immunohistochemistry.[1] Rabbit antibodies have been shown to have greater sensitivity and avidity when compared to mouse antibodies.[2,3] Additionally, some RabMAbs have been shown to be better predictors of prognosis than their MoMAb counterparts.[4] If this is so, then rabbit monoclonal antibodies may be an attractive alternative to mouse monoclonals for diagnostic use. In this study we compared RabMAbs to MoMAbs, which are currently popular in many pathology laboratories, to determine if rabbit monoclonals are a suitable substitute.

Methods

Five RabMAbs from Lab Vision Corporation (Fremont, CA) were paired with commercially available MoMAb analogs. The pairings are shown in Table 1. Each antibody was applied to a control slide previously determined to be optimal for positive reactions. All immunohistochemistry was performed in the same run under identical conditions except for the dilution of the primary antibody.

Each tissue section was pretreated by boiling for 10 minutes in citrate buffer, pH 6.0. The primary antibody was applied to each section for 30 minutes at previously determined optimal dilutions (Table 2). All primary antibodies were detected with a biotinylated polyvalent secondary antibody, streptavidin/HRP tertiary step and DAB chromagen (10 minutes each)

Rabbit Monoclonal *Mouse Monoclonal*

Cyclin D1

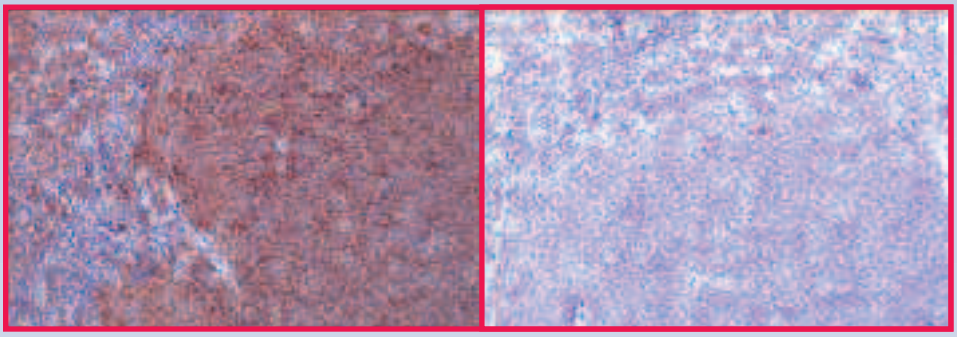

Fig. 4A. Clone SP4. Mantle cell lymphoma, 200X

Fig. 4B. Clone P2D11F11. Mantle cell lymphoma, 200X

CD3

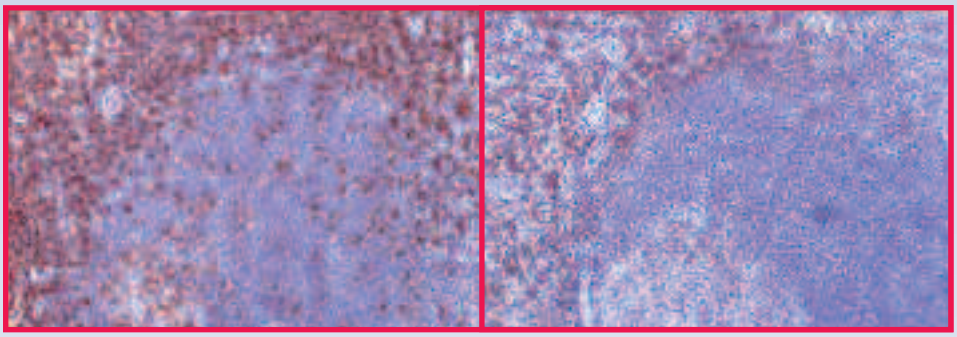

Fig. 5A. Clone SP7. Tonsil, 200X

Fig. 5B. Clone PS1. Tonsil, 200X

Table 1. — Antibody Pairing

Target	Tissue	RabMAb Clone	MoMAb Clone
ER	Breast Carcinoma	SP1	6F11
PR	Breast Carcinoma	SP2	1A6
C-erbB-2	Breast Carcinoma	SP3	CB11
Cyclin D1	Mantle Cell Lymphoma	SP4	P2D11F11
CD3	Tonsil	SP7	PS1

Table 2. — Antibody Dilutions

Target	RabMAb Clone	Dil.	MoMAb Clone	Dil.
ER	SP1	1:100	6F11	1:50
PR	SP2	1:100	1A6	1:50
C-erbB-2	SP3	1:100	CB11	1:50
Cyclin D1	SP4	1:100	P2D11F11	1:50
CD3	SP7	1:100	PS1	1:50

(all reagents from Lab Vision, Fremont, CA), and counterstained with Mayer's hematoxylin. All slides were stained on the Lab Vision 720 Autostainer.

Digital micrographs were obtained using a Micropublisher 3.0 camera. Corresponding areas of each tissue section were compared. No image processing or manipulation, beyond cropping, was performed on any image.

Results

Results obtained are shown in Figures 1-5. In all cases, the rabbit monoclonal antibody shows a stronger signal than its mouse monoclonal analog.

Conclusion

In this study, RabMAbs demonstrated stronger staining than MoMAbs. For the antibodies tested, rabbit monoclonal antibodies are a suitable alternative to mouse monoclonals in the clinical pathology laboratory.

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Some Known and Not-So-Well-Known Facts About Tissue Fixation

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Tissue fixation, as a foundation for all techniques in histology, is a subject that commands attention. The preparation of skin samples for light microscopy depends on factors of specimen handling—fixation being one of the most crucial. Laboratory practitioners and students of histology know this all too well, but it is an important point that is often neglected at clinician offices and laboratory benches.

Traditionally, fixatives have been referred to as being either coagulative or noncoagulative. However, this terminology is not only misleading, but also confusing because coagulation defines the breakdown of tissue by heat. JR Baker, a pioneer in tissue preservation, classified fixatives by their affinity to change the structure of proteins. He uses the terms coagulant and noncoagulant to denote two categories. Coagulating fixatives incorporate chemicals such as mercuric chloride in the protein molecule to form insoluble compounds. Alcohol and alcohol-based fixatives introduce nonreversible changes that result in noticeable changes in tissue.

In contrast, noncoagulating fixatives work on the rapid stabilization of proteins by cross-linking. Although details of this mechanism are unknown, the general principle is understood.

In sum, the stabilization of cells and cellular components through fixation is more accurately described as additive and non-additive fixation. Because the function of additive and nonadditive fixatives is to change the shape of tissue molecules, some enzyme reactive sites in tissue are also altered, resulting in the inhibition of tissue degradation by enzymes or autolysis.

Tissue denaturation can be explained as the drastic and irreversible change in the molecular structure of proteins, reducing the solubility of cellular components and preventing crystallization. Additive fixation, therefore, is the denaturation of tissue through the addition of the molecular components of the fixative into the tissue proteins. Examples of this type of ionic fixative are Zenker's fluid, which decreases basophilia of the nucleus and increases acidophilia of the cytoplasm; Bourn's fluid, which reacts with histones (the simplest forms of proteins) to form crystalline picrates; and alcohol.

Formalin is an excellent example of a nonadditive fixative. It combines covalently with tissue amino acids at sites containing reactive hydrogens resulting in a nonaddition product called reactive hydroxy-methyl compound. These compounds are joined to form a complex network of methyl bridges between adjacent amino acids. The change in the molecule results in increased tissue firmness because of cross-links that are formed between the fixative and tissue components.

Aldehyde fixatives, such as formalin, combine with tissue molecules at those sites where reactive hydrogen atoms are present; reactive hydrogen atoms in the basic portion of the aldehyde molecule are kept uncharged by the buffer salts in the fixative. Most of the aldehyde cross-linking in tissue fixation occurs in the hydrogen-rich areas of glutamine and lysine amino acid groups.

Tissue samples fixed in fluids containing buffers with dibasic phosphate groups such as sodium sulphate are preserved more firmly than those fixed without it. These chemical groups are efficient in creating methyl bridges or cross-links that result in better support of tissue structure during preparation.

Aldehyde fixatives develop reversible cross-links between adjacent proteins, keeping tissue elements in an in vivo-like state. Soluble proteins are fixed to structural proteins, rendering the

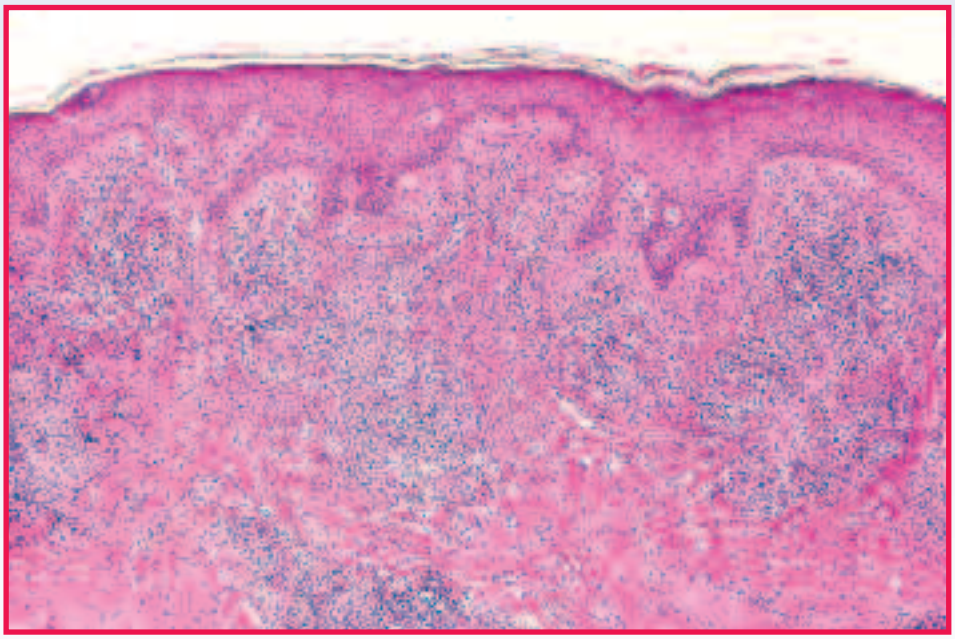

Fig. 1. A skin sample fixed in 10% neutral buffered formalin shows excellent nuclear detail, staining contrast, and good morphological preservation of tissue stroma. H&E, 200X

entire molecule resistant to subsequent maneuvers of tissue processing. Studies involving the effect of aldehydes in tissue indicate that most of the cross-links between formaldehyde and proteins are reversible; a small number of aldehyde molecules remain irreversibly attached to proteins. The majority of cross-links are easily broken down by prolonged washing (5-10 hours), rendering tissue as soft and pliable as it is in its original state before removal by scalpel. Although this is of no technical value, it helps to understand the reversible properties of the cross-linking aldehydes in formalin.

Influence of Fixation in Tissue Autolysis and Putrefaction

The removal of skin samples by scalpel initiates changes at the cellular level that eventually result in the decomposition of tissue by autolysis and putrefaction. Autolysis is the breakdown of tissue proteins by proteolytic lysosomal enzymes called cathepsins. These enzymes are released from ruptured cellular structures called lysosomes and are responsible for breaking down proteins into their individual building blocks of amino acids.

Additive and nonadditive fixation quenches the damaging effects of autolysis by changing the structure of tissue and the chemical make-up of lysosomal enzymes. Similarly, both types of fixation arrest the putrefaction of tissue by neutralizing bacterial toxins.

Optimal fixatives penetrate the specimen quickly and efficiently, preserving tissue elements such as dermal, epidermal, and adnexal structures so that they appear as true to their structures in vivo. For example, fixation preserves pathological processes within the tissue that is crucial in pattern analysis of skin disorders: basal cell carcinoma remains unaltered morphologically; malignant melanoma is preserved as it existed before excision in toto; and inflammatory diseases are set undisturbed, to appear as they were found in the patient's skin before removal. This concept is validated by examining established patterns of skin disorders. A consistent order of preservation offers a classification of skin diseases, which would be impossible if the preservation of these patterns at the cellular level is carried out haphazardly. This will help avoid, for instance, a basal cell carcinoma being indiscernible from a squamous cell carcinoma, or a melanoma being difficult to differentiate from a displastic nevus.

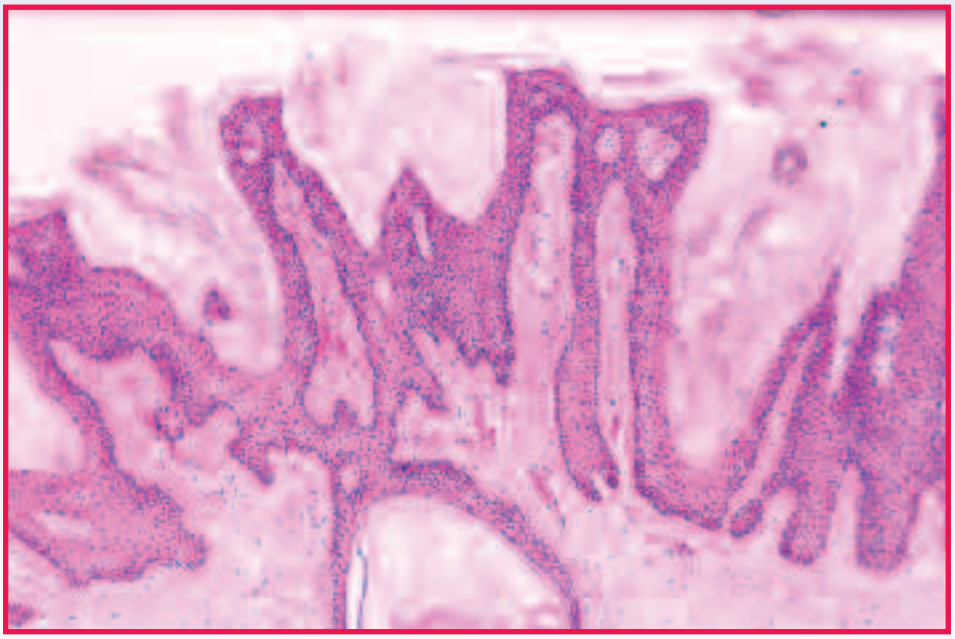

Fig. 2. A skin sample preserved in alcohol shows a pyknotic or shrinking nucleus of the epidermal keratinocytes. The condensed chromatin is shown as a structureless mass. H&E, 400X

Buffers and Hydrogen Concentrations

Before we can study the effects of acidity and alkalinity in fixation, let us understand the concept of agonal changes in tissue. Ideally, samples should be fixed completely at the moment of removal, but this is virtually impossible to achieve histologically because of factors like time and fixative penetration. From the moment the sample is removed surgically to the time the fixative reaches the core of the specimen, there is a variable span of time in which the tissue is anoxic; within 10 minutes, the lack of oxygen, or anoxia, brings about structural changes at the cellular level detectable only by electron microscopy. Within the first hour that fresh tissue remains unfixed, mitochondrial damage impacts the cell's respiratory mechanisms; enzyme depletion, such as those involved in oxidative mechanisms, are lost, and metabolic pathways are cut off, bringing profound changes to various cell organelles. In addition, cellular anoxia leads to a drastic drop in pH in the cell, resulting in the rapid deterioration of the endoplasmic reticulum, which, in turn, contributes to the loss of communication between the nucleus, messenger RNA, and the rest of the cell. Finally, rupturing lysosomes release proteolytic enzymes in the cell, which eventually leads to the breakdown of proteins and finally to decomposition or autolysis.

Hydrogen concentration determines the acidity or alkalinity of a solution, and the measurement of this level, known as pH, is adjusted by the well-balanced mixture of buffering salts. Adequate fixation of tissue morphology takes place at pH 6.9 to 7.8—the optimal range for most normal physiologic functions. Detrimental changes to tissue

structure occur when there are fluctuations of alkalinity and acidity in the fixative that fall outside the range of pH 6.9-7.8. Agonal changes incited by anoxia begin to appear when the pH within cells drops below these normal values.

Proteins are stable at a pH close to neutral; a drop in pH results in the disruption of tissue morphology. This theory can be proven experimentally by the addition of small amounts of glacial acetic acid to the fixative. In this case, the collagenous portion of a skin sample undergoes a coagulation-like process with severe obliteration of morphological detail of the connective tissue. Similar changes, but on a lesser scale, occur when formalin breaks down into formic acid, a byproduct of formalin oxidation by atmospheric oxygen. Therefore, stabilizing aldehyde fixatives with buffer solutions helps to avoid conditions favorable to the introduction of deleterious artifact in tissue. For instance, the most common buffering system used in 10% neutral buffered formalin fixation is sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4), which yields a pH range of 6.9-7.0.

Formalin was originally used in medicine as an antiseptic agent, but was eventually discontinued because of its irritating qualities. In 1893, Ferdinand Blum, a physician in Frankfurt, Germany, reintroduced the use of formalin, but this time as a preservative. He noticed the hardening effect on the skin of his own fingers when he was handling the fixative. He also demonstrated formaldehyde's ability to kill bacteria in mice infected with anthrax. Blum wrote,

"Formaldehyde in watery solution has the remarkable property to kill microorganisms with great certainty. This gradual, disinfecting quality appears to rest on unique changes of the organic material in which the

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tissue moves from their soft state of aggregation to a more resistant, harder modification. I first made this observation on my own fingers which, while working with formaldehyde, acquired a completely hardened epidermis. Then I noticed that a dissected mouse with anthrax, which has been in formaldehyde solution overnight felt like an alcohol precipitation. When I regularly placed tissues in the fluid, I became certain of the action of formaldehyde."

Laboratory workers at the time quickly recognized the importance of Blum's discovery, giving rise to the extensive literature available about fixation, much of which is still valuable today.

What Exactly Is the Solution?
Despite its universal use as a general purpose fixative, much confusion persists, however, about the exact composition of formalin. AB Lee lamented this in 1896 when he wrote:

" The already extensive literature which treats of the anatomical uses of formaldehyde is much confused by inaccurate use of the terms formol, formalin and formaldehyde. Formaldehyde is the chemical name of the compound HCOH Formol and formaldehyde is the commercial name to a 40% solution of this substance in water. Some writers use these terms indiscriminately, with the result of giving rise to much confusion."

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Chromic Acid-Schiff Stain for Fungi

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We previously reported the benefits of utilizing chromic acid oxidization when staining for fungi.[1] Chromic acid oxidation provides greater contrast between stained fungal organisms and surrounding stroma, compared to periodic acid, as used in the periodic acid-Schiff (PAS) stain. It is well documented that the PAS reaction will stain many intracellular and intercellular structures.[2] Known to be a stronger oxidizer than periodic acid, chromic acid further oxidizes aldehydes to nonreactive chemical groups, thereby reducing the binding of the Schiff molecule to structures of negligible interest.

Charles Churukian states that he uses periodic acid in place of 5% chromic acid because "chromic acid oxidation sometimes results in mucin staining so intensely that the organisms are difficult to find."[3] In our experience, this will only occur when insufficient time is spent in chromic acid. Like other chemical reactions, oxidation reactions are time and temperature dependent. The suitability of chromic acid as oxidizer in fungal stains rests with the dense carbohydrate content of fungal cell walls, which will remain available for binding to Schiff well after other carbohydrate moieties have been eliminated from staining. It should be emphasized, however, that given sufficient time, chromic acid will also eliminate reactive aldehyde groups in an organism's wall, so care must be taken to utilize a suitable incubation time for initial oxidation with this staining method.

Chromic Acid-Schiff for Fungus

Principle:
Carbohydrates in the cell walls of fungal organisms are oxidized to generate aldehyde groups that are detected with Schiff reagent. If given sufficient time, the strong oxidizer, chromic acid, will eliminate reactive aldehydes in all but the structures that have the greatest concentration of carbohydrate. This provides a cleaner background, which allows the organisms to be more readily visible.

Fixative:
10% neutral buffered formalin

Technique:
Cut paraffin sections at 4 to 5 µm
Cut kidney sections at 1 to 2 µm

Quality control:
Paraffin-embedded tissue known to be positive for any fungal species

Safety:
Chromic acid is a strong oxidizing agent and should be handled carefully. Use gloves and eye protection

Reagents:
5% Chromic acid
Chromium trioxide (CrO_3) . . . . 5 g
Deionized water . . . . . 100.0 mL

1N Hydrochloric acid
Hydrochloric acid, concentrated (specific gravity, 1.19) . . . . 83.5 mL
Distilled water . . . . . 916.5 mL

Add the acid to the water and mix well.

Schiff reagent
Distilled water . . . . . 800.0 mL
Basic fuchsin . . . . . 4.0 g
Sodium metabisulfite . . . . . 4.0 g
1N hydrochloric acid . . . . . 80.0 mL

Heat water to the boiling point. Remove from flame, add basic fuchsin, and heat solution to the boiling point. Cool the solution to 50°C and then filter. Add 80 mL

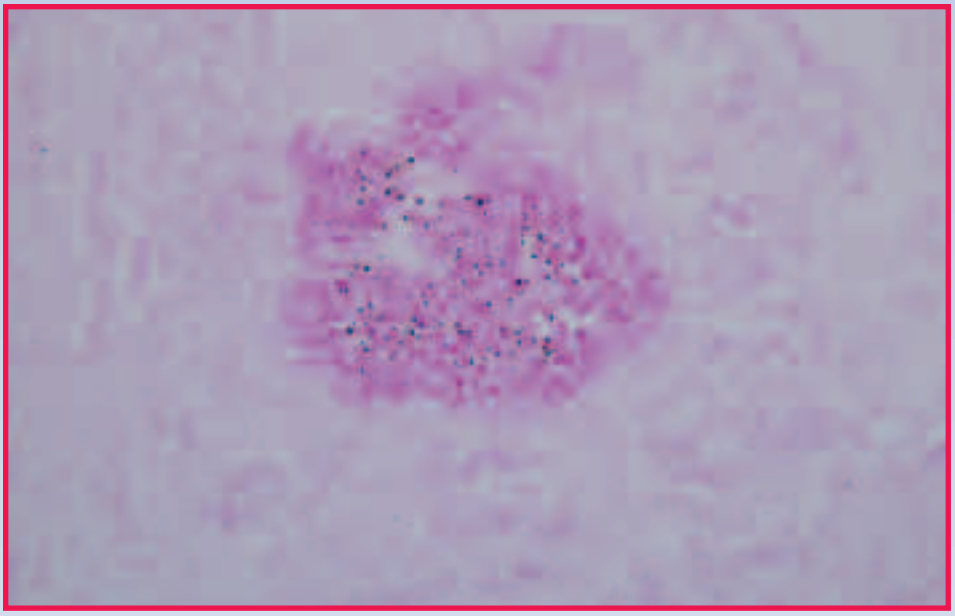

Fig. 1. Fungal organism in lung tissue demonstrated with chromic acid-Schiff stain, 10 min oxidation in 4% chromic acid at 60°C. 200X

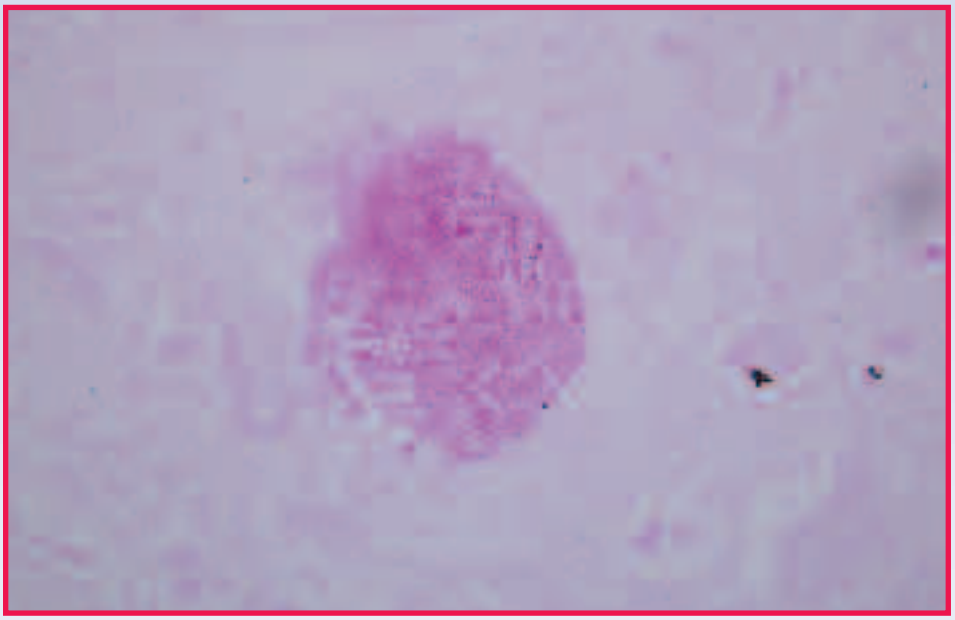

Fig. 2. Fungal organism in lung tissue demonstrated with chromic acid-Schiff stain, 1 hr oxidation in 4% chromic acid at room temperature. 200X

of 1N HCl, cool completely, and add 4.0 g of sodium metabisulfite. Let the solution stand in the dark overnight; it should turn light amber. Add 2.0 g of activated charcoal and shake for 1 minute. Filter the solution and store in the refrigerator.

Test for quality of Schiff Reagent[4]
Place 10 mL of 37% to 40% formaldehyde in a beaker or Erlenmeyer flask. Add drops of Schiff reagent. If the solution *rapidly* turns reddish purple, it is good to use. If reaction is delayed and the resultant color is a deep blue-purple, the solution should be replaced.

Procedure:

1. Deparaffinize and hydrate slides to distilled water.
2. Place sections in 5% chromic acid solution for 8-10 minutes at 60°C* or 1 hour at room temperature.
3. Wash slides in three changes of deionized water.
4. Place sections in Schiff reagent at room temperature for 15 minutes. Be certain that solution has been brought to room temperature prior to staining.
5. Wash in running tap water for 15 minutes to develop full color.
6. Counterstain in Gill's hematoxylin for 2 minutes.
7. Wash sections well. Dip in ammonia water 2 minutes to blue.
8. Dehydrate with 95% and absolute alcohol, clear with xylene, and mount with synthetic resin.

* Chromic acid may arc in a microwave oven, therefore, a conventional oven or water bath is recommended for heating this solution.[5]

Results
Mycelia, conidia, and yeast forms will stain a deep rose to purple.

Conclusion
The following points highlight our observations:

1. Insufficient oxidation time in chromic acid may lead to increased background staining. Prolonged exposure to chromic acid, especially under heat, may cause reduced staining of fungal organisms.
2. In our hands, as the chromic acid solution ages, its oxidative ability appears to diminish. We prefer to make fresh solution every 2 weeks. If using older solutions, incubation times may need to be increased.
3. The sulfite rinses are essential to removing any unbound leucofuchsin following exposure to the Schiff reagent.
4. Thorough washing in tap water after the sulfite rinses is essential for adequate color development.

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Prolonged Storage of Silver-Stained Slides in Xylene May Cause Fading

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During the preparation of stained slides for her ASCP certification exam late one evening in the laboratory, an exhausted employee left her Steiner-stained sections in the last dish of xylene in the lab's staining setup. The stain had been performed to demonstrate *Helicobacter pylori,* using a known positive control tissue. Upon arrival the next day, she discovered that the stained sections had almost completely faded and could not be used for her exam.

It is not uncommon for labs to occasionally allow stained slides to sit in xylene overnight. In most instances this occurs without incident. Anyone who has accidentally overstained slides with a silver technique can attest that removal of the extra silver requires deliberate steps that aren't always successful. You can imagine that the accidental removal of silver, as occurred in this case, was really quite a surprise. We offer this information in the hope that it will enable others to avoid this problem.

Prior to this episode, we were unaware of any mention in the literature of stains fading in xylene. However, we discovered remarks offered by Winsome Garvey in an article entitled "Silver impregnation techniques to identify spirochetes and other bacteria," where it was noted that

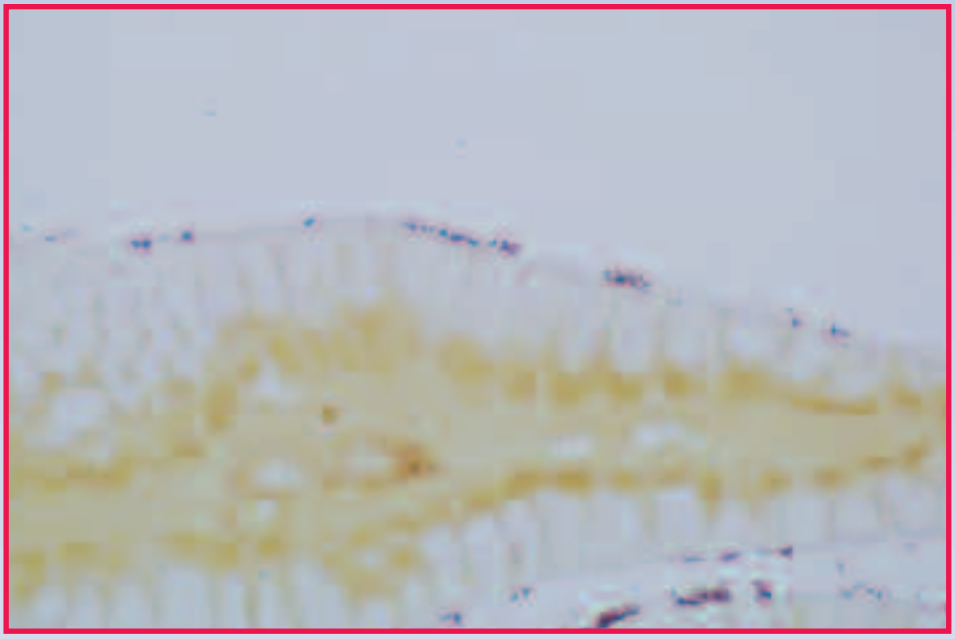

Fig. 1. Positive control tissue demonstrating *Helicobacter pylori* in gastric tissue. Steiner method, 600X

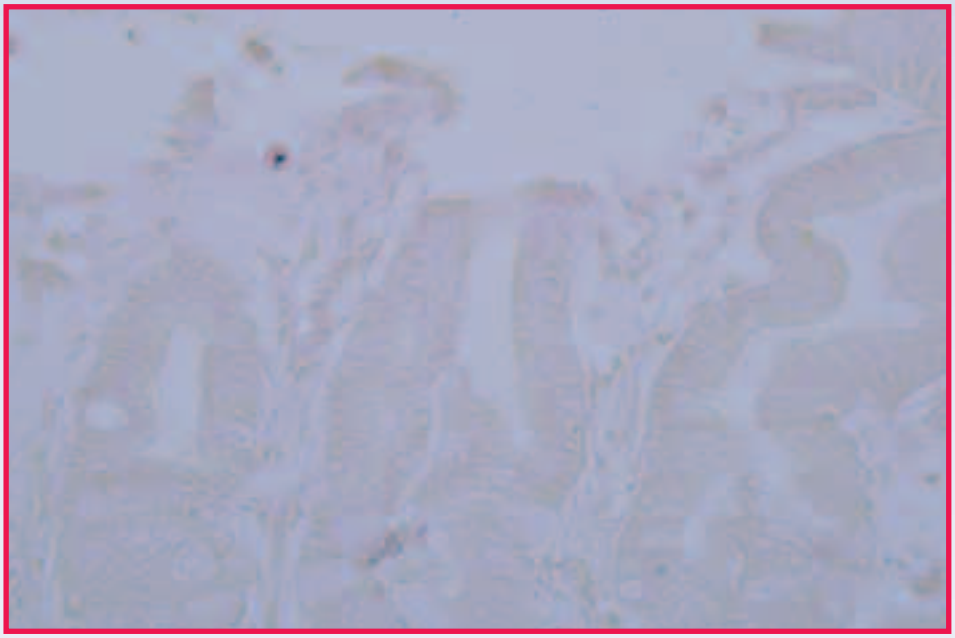

Fig. 2. *Helicobacter pylori* positive control in gastric tissue stained with the Steiner method, allowed to sit in xylene overnight, which resulted in severe fading. 400X

"…clearing of (silver-stained) sections before coverslipping must be performed in a fresh change of clearing agent to avoid exposure to oxidants, which can cause fading of silver. Some laboratory workers use the same clearing agent for all stained sections, not realizing that oxidants may be carried over from other stains."[1]

Reference

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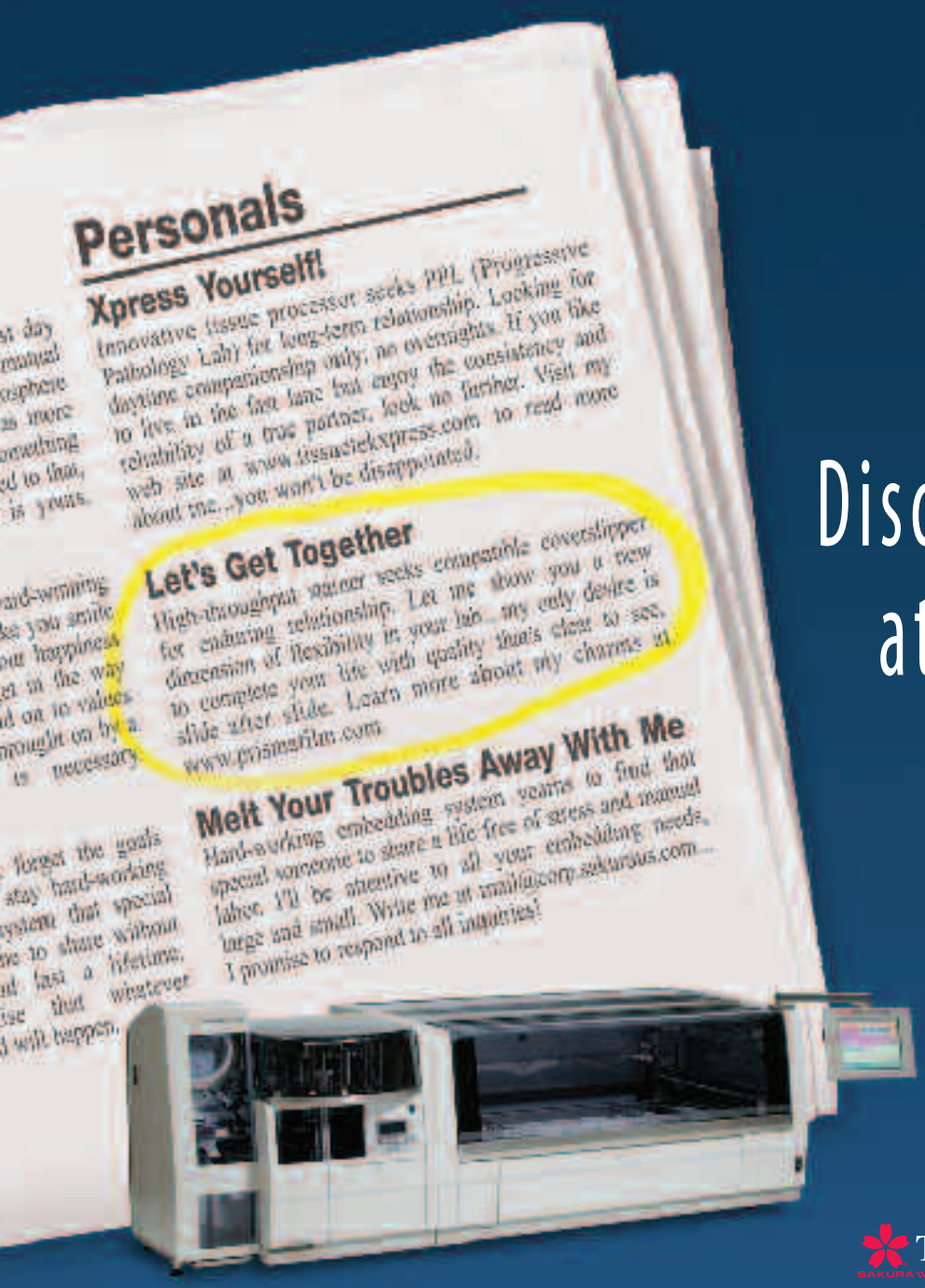

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Speaker: Ethel Macrea, HT(ASCP), Q-IHC
Ventana Medical Systems Inc.,
Tucson, AZ

January 20
University of Texas Health Sciences Ctr/ San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"The Shipping Regulation Wheel of Fortune—Where Will It Stop, Category A, Category B or Exempt Specimens?"**
Speaker: Linda Durbin, President
EXAKT Technologies, Inc.
Oklahoma City, OK

February 16
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221 histo@nsh.org
Title: **" Immunohistochemical Staining of Cytokeratins (CK)"**
Speaker: Mahanpal Singh Dulai, MD
William Beaumont Hospital
Royal Oak, MI

February 17
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Title: **"Mohs Histographic Surgery for Histologists"**
Speaker: Clifford M. Chapman, MS, HTL(ASCP), QIHC
Pathology Services, Inc.
Cambridge, MA

March 3-4
Indiana Society for Histotechnology
Site: Indianapolis, IN
Contact: LaDonna Elpers (812) 985-5900 ext 128
Email: lelpers@bioanalytical.com

March 11
Arkansas Society of Histotechnology
Site: Harrison, AR
Contact: Melissa Hill (479) 442-0144
Email: mkh@nwapath.com

March 15
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221 histo@nsh.org
Titlte: **"Spill Kits and Formaldehyde Neutralization"**
Speaker: Peggy A. Wenk, HTL(ASCP)SLS
William Beaumont Hospital
Royal Oak, MI

March 17
University of Texas Health Sciences Ctr/ San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Basic Principles of Fixation"**
Speaker: Barry Rittman, PhD
University of Texas Health Science Center
Houston, TX

March 30- April 1
Texas Society for Histotechnology
Site: Omni Bayfront & Marina Towers
Corpus Christi, TX
Contact: Veronica Davis
Email: veronida@baylorhealth.edu

April 1
Nebraska Society for Histotechnology
Site: Mahoney State Park, NE
Contact: Judy Webb
Email: jwebb01@jpshealth.org

April 12
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221 histo@nsh.org
Title: **"Staining for *Helicobacter pylori*"**
Speaker: Sandy Wilkins, HT(ASCP)
Michigan Institute of Urology
St. Clair Shores, MI

April 21
University of Texas Health Sciences Ctr/ San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Microwave Technology Today"**
Speaker: Donna Willis, HT/HTL(ASCP)
Site: Harris Methodist Hospital
Ft. Worth, TX

April 21-22
North Carolina Society of Histopathology Technologists
Site: Marriott Airport, Greensboro, NC
Contact: Delorise Williams
Email: dwilliams@ciit.org

April 27-28
Wisconsin Histology Society
Site: LaCrosse, WI
Contact: Maureen Decorah
Email: decorah@rarc.wisc.edu

April 29-30
Region 1 Symposium
Site: Saratoga Springs, NY
Contact: Jason Burrill
Email: jason.burrill@crl.com

May 5-6
Michigan Society for Histotechnology
Site: Doubletree Hotel, Novi, MI
Contact: Paula Bober
Email: pbober@dmc.org

May 5-7
Florida Society for Histotechnology
Site: Embassy Suites
Deerfield Beach, FL
Contact: Jerry Santiago
Email: santij1@earthlink.net

May 17
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Title: **"Communication Styles—Working With Difficult People"**
Speaker: Amy Dixon, William Beaumont Hospital
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May 18-19
Illinois Society for Histotechnology
Site: Lisle, IL
Contact: Jane Chladny
Email: jchladny@cvm.uiuc.edu

May 19
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Title: **"Decalcification Techniques & Regulatory for the Non-Clinical Histology Laboratory "**
Speaker: Diane L. Sterchi, MS, HT/HTL(ASCP), EM (MSA)

June 7
Georgia Society for Histotechnology
Site: August, GA
Contact: Connie Wavrin
Email: cwavrin@labmd.org

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Contact: Jerome Jasso

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Email: sbreeden@nmda.nmsu.edu

July 19 — **NSH Teleconference 1:00 pm Eastern Time**
Contact: (301) 262-6221 histo@nsh.org
Title: **"Troubleshooting Silver Stains"**
Speaker: Joan Vesey HT(ASCP)
Richard-Allan Scientific
Kalamazoo, MI

July 21 — **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Identification of Common Pathogenic Fungi and Parasites in Histologic Sections"**
Speaker: Elizabeth A. Sheppard, MBA, HT(ASCP)
Ventana Medical Systems, Inc.
Tucson, AZ

August 18 — **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Regulatory Issues for the Non-Clinical Histology Laboratory"**
Speaker: Diane L. Sterchi, MS HT/HTL (ASCP), EM (MSA)

September 9-14 — **NATIONAL SOCIETY FOR HISTOTECHNOLOGY SYMPOSIUM/CONVENTION**
Site: Phoenix, AZ
Contact: Aubrey Wanner, NSH Office
Tel: (301) 262-6221
Fax: (301) 262-9188
Email: Aubrey@nsh.org

September 15 — **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Issues in Processing Breast Specimens"**
Contact: I-Tien Yeh, MD
University of Texas
Health Science Center
San Antonio, TX

October 20 — **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Troubleshooting Special Stains Techniques"**
Speaker: Jerry Santiago, BS, HTL(ASCP)QIHC
Florida Community College
Jacksonville, FL

November 15 — **NSH Teleconference 1:00 pm Eastern Time**
Contact: (301) 262-6221 histo@nsh.org
Title: **"The Science of Tissue Processing"**
Speaker: Sherri L. Anderson, HTL(ASCP)
Thermo Electron Corporation
Pittsburgh, PA

November 17 — **University of Texas Health Sciences Ctr/ San Antonio**
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Reduction of Medical Errors: Applications for the Histology Laboratory"**
Speaker: Terri Braud, HT(ASCP)
University of Virginia Medical Center
Charlottesville, VA

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