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Technical Bulletin for Histotechnology

Effects of Fixation and Decalcification on Kappa and Lambda Staining by Immunohistochemistry

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Fig. 1. Formalin-fixed tonsil tissue stained with anti-kappa anibody and different decal solutions: (A) Formical-4™; (B) RDO; (C) Immunocal™; (D) Versenate. 10X

Introduction

10% neutral buffered formalin (NBF) has been the routine fixative and gold standard in the histology laboratory for many decades. However, sometimes there is a need to select a fixative other than 10% NBF. One such reason is the need to improve nuclear detail and chromatin pattern in lymphoid tissue. Another reason is health concerns for lab personnel due to the toxicity of formalin-based fixatives. In recent years we have seen a move away from formalin-based fixatives and an increased interest in formalin substitutes.

Immunohistochemical staining for kappa and lambda light chains is often performed on lymphoid tissue. Staining for kappa and lambda light chain expression is notoriously problematic—some might say finicky. One complication is the need for
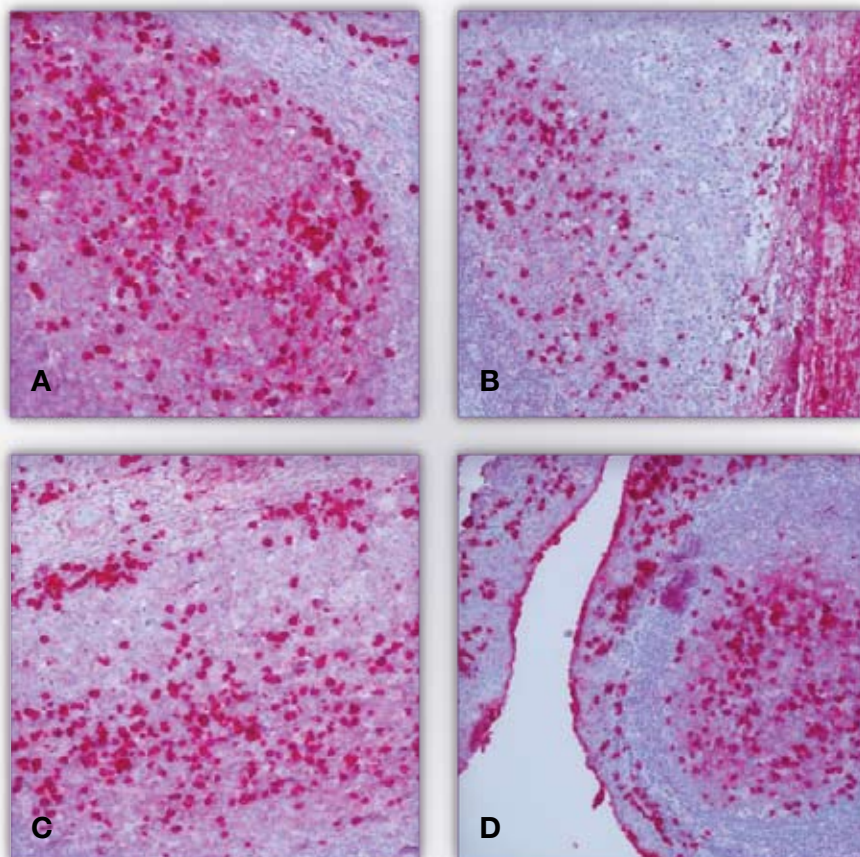
Effects of Fixation on Kappa and Lambda Staining by IHC

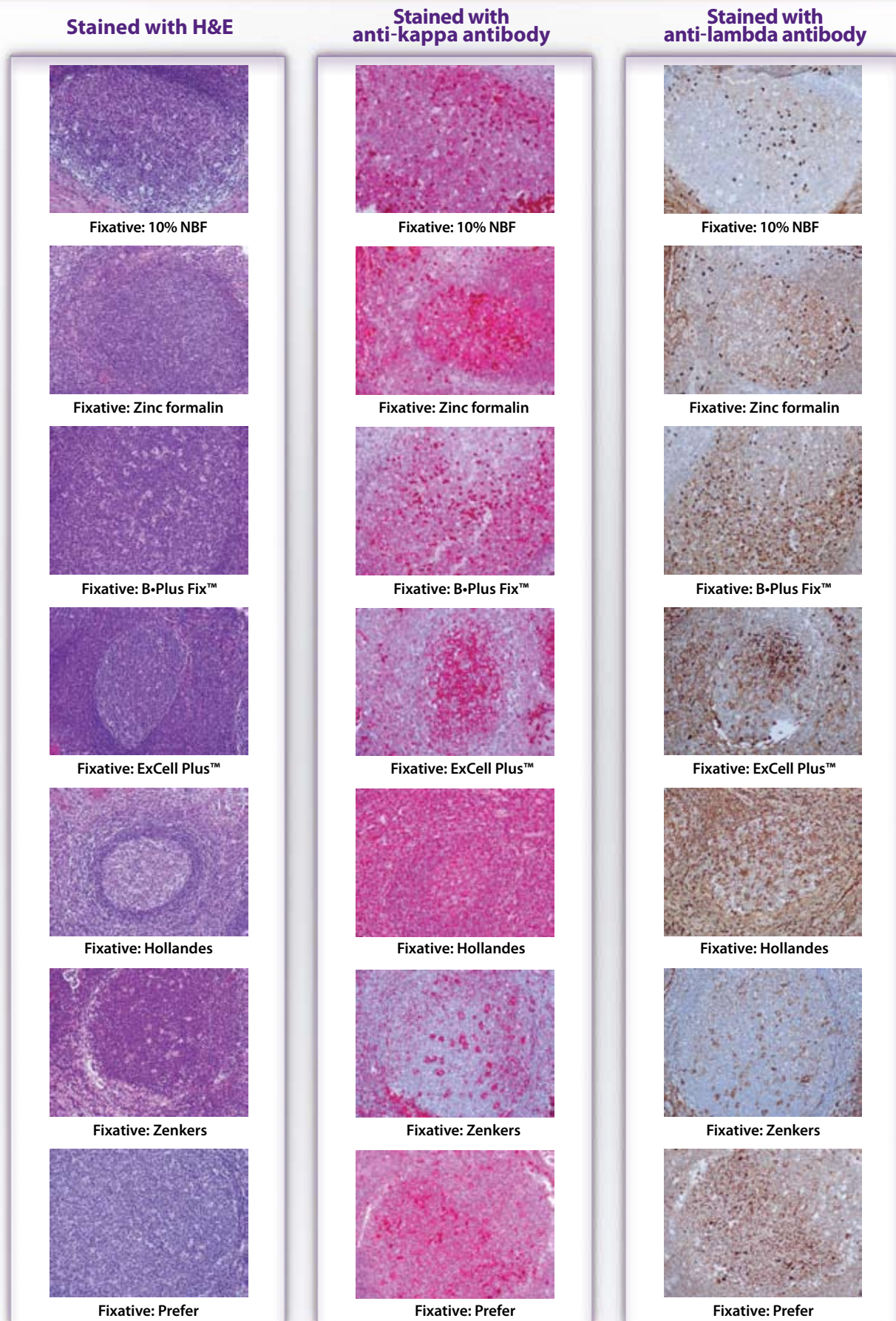| Stained with H&E	Stained with anti-kappa antibody	Stained with anti-lambda antibody
Fixative: 10% NBF	Fixative: 10% NBF	Fixative: 10% NBF
Fixative: Zinc formalin	Fixative: Zinc formalin	Fixative: Zinc formalin
Fixative: B•Plus Fix™	Fixative: B•Plus Fix™	Fixative: B•Plus Fix™
Fixative: ExCell Plus™	Fixative: ExCell Plus™	Fixative: ExCell Plus™
Fixative: Hollandes	Fixative: Hollandes	Fixative: Hollandes
Fixative: Zenkers	Fixative: Zenkers	Fixative: Zenkers
Fixative: Prefer	Fixative: Prefer	Fixative: Prefer

Fig. 2. Effects of 7 different fixatives on tonsil tissue stained with H&E or with kappa and lambda staining by IHC. 10X

Effects of Decalcification on Kappa and Lambda Staining by IHC

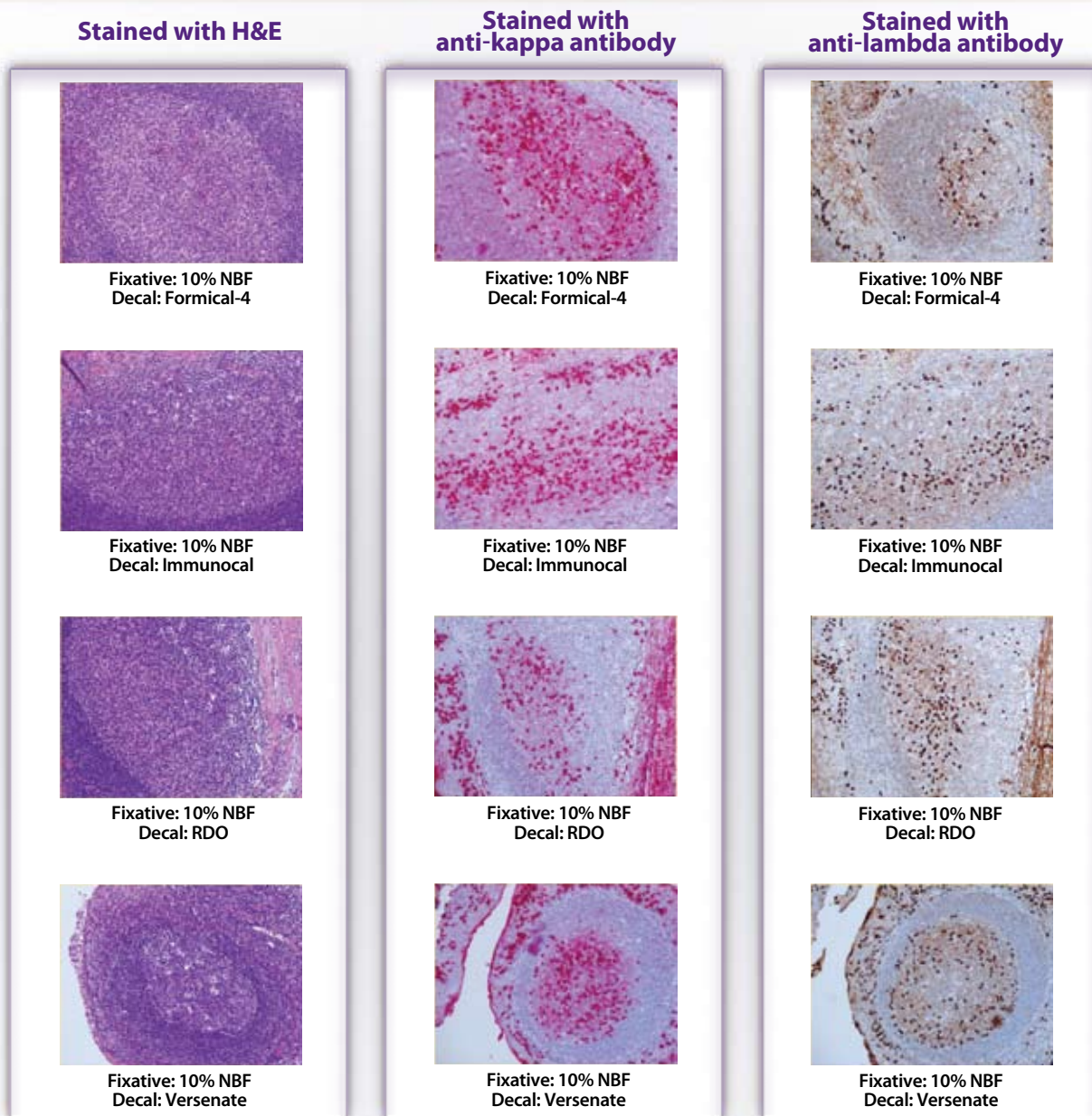| Stained with H&E	Stained with anti-kappa antibody	Stained with anti-lambda antibody
Fixative: 10% NBF **Decal: Formical-4**	**Fixative: 10% NBF** **Decal: Formical-4**	**Fixative: 10% NBF** **Decal: Formical-4**
Fixative: 10% NBF **Decal: Immunocal**	**Fixative: 10% NBF** **Decal: Immunocal**	**Fixative: 10% NBF** **Decal: Immunocal**
Fixative: 10% NBF **Decal: RDO**	**Fixative: 10% NBF** **Decal: RDO**	**Fixative: 10% NBF** **Decal: RDO**
Fixative: 10% NBF **Decal: Versenate**	**Fixative: 10% NBF** **Decal: Versenate**	**Fixative: 10% NBF** **Decal: Versenate**

Fig. 3. Effects of 4 different decal solutions on tonsil tissue that was fixed in 10% NBF and stained with H&E or with kappa and lambda staining by IHC. 10X

decalcification on some lymphoid tissue often stained for kappa and lambda, such as bone marrow core biopsies. The more common fixatives currently in use have different effects on the various tissues under study and may impart different properties to the protein targets in question.

This article explores the effects of various fixation and decalcification solutions on immunohistochemical staining for kappa and lambda light chains in lymphoid tissue.

Materials and Methods

Fresh tissue samples of tonsil were collected and sectioned into 2-3 mm slices and placed in seven different fixatives for the appropriate times according to either standard practice or the manufacturer's instructions. The samples were then routinely processed and infiltrated with paraffin. A final multitissue block was created from samples of each tissue fixed in the various fixatives (Fig. 2).

The decalcification study was performed on tissue that was first fixed in 10% neutral buffered formalin (NBF) for 8 hours. The tissues were then placed in one of four decal solutions, according to the manufacturer's recommended time. A final multitissue block was created from the various decalcified tissue samples (Fig. 3).

Hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining for kappa and lambda light chains were performed on the BenchMark® XT staining platform from Ventana Medical Systems (Tucson, AZ).

Table 1. Tissue Fixation

Fixative	Fixation Time
10% NBF	8 hours
Zinc formalin	8 hours
B•Plus Fix™*	4 hours
Zenkers	4 hours
Prefer†	4 hours
ExCell Plus™‡	4 hours
Hollandes	4 hours

*B•Plus Fix™ is a trademark of BBC Biochemical (Mount Vernon, WA).
†ExCell Plus™ is a trademark of American Master*Tech Scientific, Inc. (Lodi, CA).
‡Prefer Fixative, Anatech Ltd. (Battle Creek, MI).

Table 2. Tissue Decalcification

Decal Solution	Decal Time
Immunocal™*	30 minutes
Formical-4™†	30 minutes
Versenate‡	Overnight
RDO§	30 minutes

*Immunocal ™ is a trademark of Decal Chemical Corp. (Tallman, NY).
†Formical-4™ is a trademark of Decal Chemical Corp. (Tallman, NY).
‡Versenate Chelating Agent, American Master*Tech Scientific, Inc. (Lodi, CA).
§RDO Rapid Decalcifier, Apex Engineering Product Corp. (Aurora, IL).

The same staining protocol conditions were used across each of the various fixatives and decal solutions. We did not attempt to optimize the protocol conditions to compensate for the differences in protein preservation. All antibodies, detection chemistries, and ancillary reagents were from Ventana Medical Systems.

Results

The images in Fig. 2 demonstrate the results of tonsil tissue fixed in seven different fixatives and stained with H&E, as well as lambda staining with DAB and kappa staining with alkaline phosphatase red detection chemistries. The images in Fig. 3 compare four different decalcification solutions also stained with H&E, as well as lambda staining with DAB and kappa staining with red chromogen. H&E staining was performed on all tissue samples. Images of H&E staining are provided for reference.

Discussion

In this experiment we used a staining protocol whose conditions were optimized to provide primarily plasmacytoid staining with some germinal center staining on 10% NBF-fixed tissue. Using the same protocol conditions across multiple fixative and decalcification solutions, we were able to demonstrate kappa and lambda light chain expression across all fixatives and decal solutions.

The amount of light chain staining, however, varied among the different fixatives with the non-formalin-based fixatives demonstrating the greatest amount of lymphocytic and interstitial staining. All fixatives, including formalin-based fixatives, were able to demonstrate plasmacytoid staining. The staining results using Hollandes and Prefer fixatives provided the greatest challenge with regard to staining interpretation.

All decalcification solutions, in combination with 10% NBF fixation, were able to demonstrate plasmacytoid staining. Immunocal and Formical-4 yielded better morphology and immunostaining of the mantle zone lymphocytes. Staining of these cells was significantly diminished with the use of RDO and Versenate as decalcification solutions, when no other variables were changed.

Using the same protocol conditions across multiple fixatives and decalcification solutions, we were able to demonstrate kappa and lambda expression with all fixatives and decal solutions. Therefore, it is important to consider pathologist preference, cell type and its optimal staining requirements, as well as health risks to lab personnel when choosing which fixatives and decal solutions to use.

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Reclaiming Maximum Antigenicity From Previously Stained Samples: a Recapture Method for Immunohistochemistry

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Abstract

Heat-induced epitope retrieval (HIER) has contributed to the optimization of many antigens that might never have been visualized without this technique. Over time, the benefits of using HIER to access antigens have prompted replacing most enzyme retrieval methods with antigen recovery. Understanding the many variables, such as time, temperature, and pH of the retrieval solution, has greatly influenced the use of antigen retrieval (AR) methods for visualization of epitopes that require unmasking.

In recognizing the value of the HIER technique, our core lab questioned whether it might be used to recapture antigenicity in previously stained immunohistochemistry samples. As part of an internal investigation to examine variable staining in terminal deoxyribonucleotide transferase (TdT), we applied a number of retrieval options.

We found that by running the sample back to the AR step and repeating the specific retrieval sequence, we could achieve consistent results, not only for the TdT, but also with a variety of previously stained but variable antibodies.

Introduction

Enzyme pretreatment of tissue samples to access antigen binding sites is the earliest type of retrieval method. Also known as proteolytic enzyme digestion, antigens blocked by fixation in cross-linking fixatives, such as formalin, often require digestion of cross-links to open access to binding sites. Enzyme-induced epitope retrieval (EIER), first described by Huang et al in 1976, uses enzymes such as ficin, trypsin, or proteinase K to dissolve blocked sites.[1] EIER still remains an important method for providing complete retrieval for some antigen sites.

Heat-induced epitope retrieval, more recently called heat-induced antigen retrieval (HIAR), was first described in 1991 by Shi et al, who introduced antigen recovery in water.[2] Since then, HIAR has largely replaced EIER for recovery of most antibodies. Using a combination of buffers, pH, and time, we have learned to unmask a great number of previously cross-linked antigens. Whether we use a water bath, microwave, steamer, or pressure cooker to achieve heating, we are able to successfully unmask antigens from the effects of formalin cross-linking, while maintaining the morphology of formalin-fixed tissue.

However, HIAR can also be plagued with problems. Specifically, high temperature exposure can cause damage to some antigens; use of heated buffers can result in tissue detachment from the glass slide or folding; and uncontrolled retrieval can cause incomplete or over-retrieved samples, yieding confusing or inaccurate results.[3] It is this last problem, variability in staining, that we found most troubling, and which prompted us to seek some remedy.

In conferring with several other hospital labs we learned that many were also struggling with variable staining for the TdT marker.

Often, we have little control over the preparation of tissues we receive as referral samples from other facilities. We know that many problems associated with variable staining are often a result of improper fixation and processing,[4] therefore we began our investigation by eliminating these as a source of the staining problem.

We determined that incomplete deparaffinization of samples was often responsible for variable staining in the TdT samples. This problem can be attributed to the melting point of the embedding paraffin that is used. We know that deparaffinization is important to access the antigen sites, but we noted some antigens required absolute deparaffinization to provide stable and complete antigen retrieval.[5]

Using paraffin with high melting points or those containing plasticizers can cause resistance to complete removal, especially if xylene substitutes are used in the initial deparaffinization. We observed that the use of xylene to deparaffinize always improved the signal. We explored whether rerunning the variable samples back through xylene and repeating the AR procedure on a previously stained sample would improve staining results. By taking the samples back through multiple xylene rinses, we might uncover cross-linked sites not accessed in the original staining procedure and have better or full recovery of the sample antigenicity. Our major concerns with this procedure included completion without losing the original signal, over-retrieving, or damaging the tissue morphology.

Materials and Methods

Recovery Protocol
Slides from a sample that had been embedded with a high temperature paraffin, deparaffinized in a xylene substitute, and presented a poor signal for TdT were placed in xylene to remove the cover glass. Then they were run back through 6 successive xylene rinses to remove the residual mounting

media as well as any remaining paraffin. The slides were rehydrated through graded alcohols to deionized water with the following protocol.

Primary Protocol

Formalin-fixed paraffin-embedded (FFPE) sections of thymus were cut at 5 µm on a rotary microtome, heat immobilized for 1 hour on Superfrost® slides (Thermo Fisher, Pittsburgh, PA); 3 slides with controls.

1. All slides were deparaffinized in xylene substitute and hydrated to deionized water.

2. All slides were initially retrieved in citrate buffer at pH 6.0 for 20 minutes in a Biocare Decloaker pressure cooker (Biocare Medical, Concord, CA) and then cooled for 20 minutes.

3. After appropriate blocking of endogenous proteins, the slides were placed on a Microm HMS710i immunostainer; the primary antibody, TdT (NeoMarker, MS-1105-S) was diluted 1:60 in 1X TBS (Tris buffered saline) with 0.05% Tween-20, 1 hour incubation at room temperature.

4. Slides were developed using UltraVision LP-HRP-Polymer detection system and DAB substrate (LabVision, Fremont, CA).

5. All slides were counterstained in Mayer's hematoxylin (Rowley Biomedical, Danvers, MA).

6. Slides were dehydrated and cleared in xylene substitute and mounted in Permount®.

Result: *variable staining.*

Fig. 1. Thymus tissue stained with TdT (A) on the first run; (B) on the recovery run; (C) negative for TdT on the recovery run. 20X

All photographs taken with Olympus BX51.
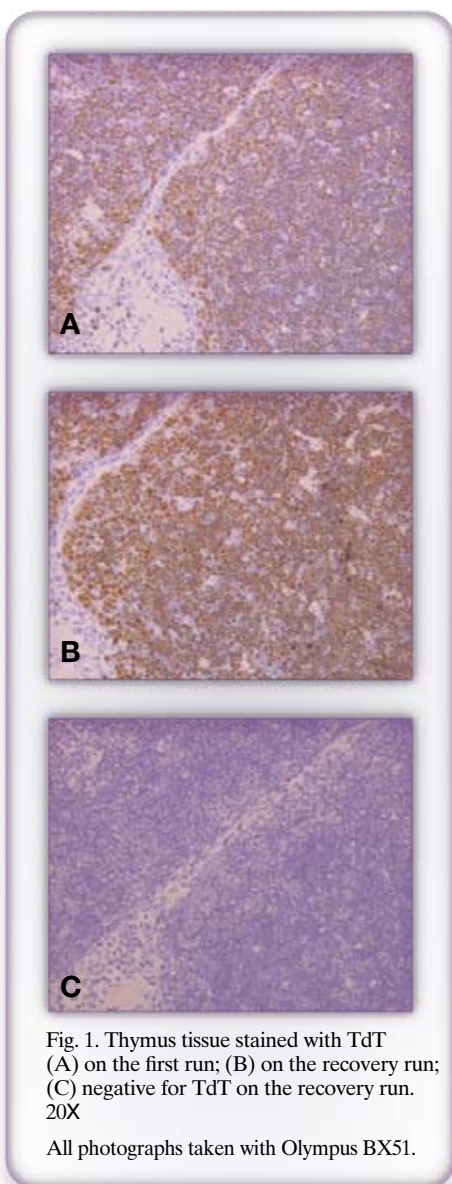
Secondary protocol

Antigen recapture from previously stained slides

1. Slides were placed in xylene to remove the cover glass.

2. After removal of the cover glass and adequate removal of all remaining mounting medium, the slides were rinsed through 6 xylene washes for 5 minutes each to ensure removal of any remaining paraffin.

3. The slides were rehydrated through graded alcohols to deionized water.

4. AR was repeated with citrate buffer in the decloaker (step 2 in the primary protocol) and the slides allowed to cool completely to room temperature before moving ahead with the immunohistochemistry (IHC) (steps 3-6 in the primary protocol); slides were then remounted in Permount.

Results

After several trials of the repeat TdT protocol to confirm results, we found that by running the samples back through several xylene rinses and repeating the previous AR protocol, we safely reclaimed antigen sites that had remained blocked by incomplete deparaffinization in the initial run. There was no significant damage to the tissue morphology when handled carefully through all repeat steps, and the signal was markedly improved, with minimal or no increase in background staining (Fig. 1).

The authors also used this same method in opening additional sites for better staining of catenin (Fig. 2). Catenin is another antibody that can yield variable staining. It is believed that incomplete deparaffinization may interfere with obtaining a good signal. We are currently investigating the use of this method for other antigens that are problematic.

Fig. 2. Liver tissue stained with catenin (A) on the first run; (B) on the recovery run; (C) negative for catenin on the recovery run. 20X

All photographs taken with Olympus BX51.
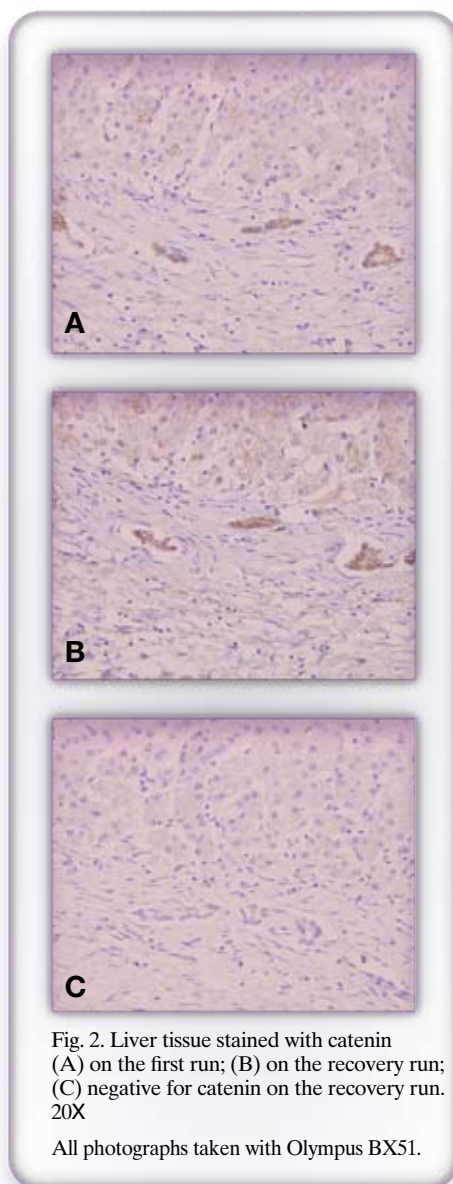
Discussion

There are many potential causes of variable staining in immunohistochemistry. In our experience, incomplete deparaffinization of tissues to be stained is a predominate cause of variable staining. A superior paraffin solvent such as xylene should be used for more thorough removal of paraffin from tissue sections prior to staining. In our experience, xylene substitutes have caused incomplete access of antibodies to their binding sites. This work illustrates that blocked antigen binding sites in sections exhibiting variable staining with IHC can be reclaimed without damaging the tissue morphology or creating nonspecific background staining. We believe that this simple method to reclaim more antigen sites in previously stained samples is but another step in refining the IHC technique.

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Acknowledgments

Automating a Reticular Fiber Stain: Challenges and Benefits

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Abstract

Few stains in the histology laboratory present as much of a challenge as silver stains. We have chosen to look at silver staining techniques for reticular fibers in the Gordon and Sweets method, as this is the method we utilize most often in our laboratory. This is our primary method for visualization of reticular fibers in human liver samples.

This method presents very specific challenges. As with all silver stains, no metal may be used, the ammoniacal silver solution must be made fresh just prior to use, and during the differentiation of the stain there is room for variation in dipping speeds from individual to individual. These challenges become even more cumbersome in the automation process for the following reasons: traditional adaptors for automated stainers are made out of metal and touch the solutions during the staining process; solutions tend to sit during the length of the staining process and are not made just prior to use; and the dipping speed tends to be relatively slow, which may cause overdifferentiation of the slides.

Automation of a silver stain may present several challenges, but the singular reward outweighs them all—more consistent results by reducing human variability. In this report we describe our efforts to overcome the stated obstacles of automating the Gordon and Sweets silver staining technique for reticular fibers.

Materials and Methods

Commercially obtained slides of formalin-fixed paraffin-embedded (FFPE) human liver (cat #4620A, Newcomer Supply, Middleton, WI) were utilized as positive controls. Slides were evenly distributed among plastic staining racks (cat #4768, Sakura Finetek, Torrance, CA) and metal adaptors (Sakura cat #2209) with the Sakura DRS-601™ Autostainer (Sakura Finetek, Torrance, CA).

Reagents for the Gordon and Sweets Reticulum Procedure (cat #k082, Polyscientific R&D Corp, Bayshore, NY) were utilized.

Sodium thiosulfate was used to remove any unreacted silver after toning in gold chloride.[1]

All reagents in the staining kit come premixed except for potassium permanganate working solution, silver nitrate working solution, and sodium thiosulfate 5% (cat #SX0820-1, EMD Chemicals Inc., Gibbstown, NJ). All other reservoirs (Sakura cat #2203) in the instrument should be filled **prior** to making potassium permanganate working solution and silver nitrate working solution.

Table 1. Placement of Reagents in Autostainer

Reagent	Reservoir Number	Solution Volume
Xylene	1	500 mL
Xylene	2	500 mL
Reagent alcohol 100%	3	500 mL
Reagent alcohol 100%	4	500 mL
Reagent alcohol 95%	5	500 mL
Reagent alcohol 95%	6	500 mL
Potassium permanganate working solution	12*	360 mL
Reverse osmosis/deionized water (RO/DI water)[†]	11	360 mL
Oxalic acid 1%	10	500 mL
Ferric ammonium sulfate 2.5%	9	500 mL
RO/DI water[†]	8	500 mL
Silver nitrate working solution	7[‡]	360 mL
RO/DI water[†]	13	360 mL
Formaldehyde 37%	14	360 mL
RO/DI water[†]	15	500 mL
Gold chloride 0.5%	16	500 mL
RO/DI water[†]	17	500 mL
Sodium thiosulfate 5%	18	500 mL

*Solution should be mixed fresh as follows: 380 mL potassium permanganate 0.5% aqueous to 20 mL of sulfuric acid 3% aqueous. Add 360 mL to the reservoir and dispose of the remaining 40 mL according to local hazardous waste regulations.

[†] All reservoirs containing deionized water rinses are collected and disposed of in accordance with local hazardous waste regulations.

[‡] Solution should be mixed fresh in acid-cleaned glassware as follows: place 25 mL of silver nitrate 10% aqueous in an Erlenmeyer flask (100 mL). Add concentrated ammonium hydroxide drop by drop while constantly swirling solution. A grayish brown precipitate will begin forming then turn black and ultimately clear. Add 25 mL of sodium hydroxide 3% aqueous and a black precipitate will form. Again add concentrated ammonium hydroxide drop by drop while constantly swirling solution until solution is nearly clear with a slight smoky color remaining.[2] Transfer solution to a 500 mL graduated cylinder and add deionized water until a total volume of 400 mL is obtained. Add 360 mL to the reservoir and dispose of the remaining 40 mL according to local hazardous waste regulations.
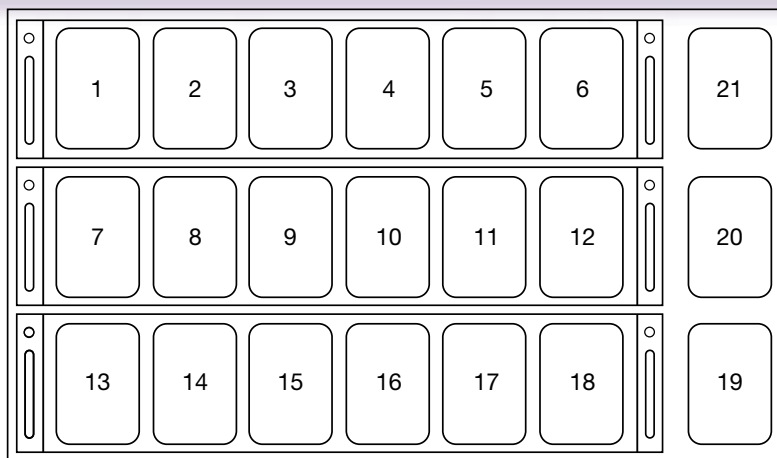
Fig. 1. Reagent reservoir arrangement for the Sakura DRS-601 Autostainer.[3]

Table 2. Automated Gordon and Sweets Protocol

Step Number	Reservoir Number	Immersion Time
1	1	5'00"
2	2	5'00"
3	3	2'00"
4	4	2'00"
5	5	2'00"
6	6	2'00"
7	21*	1'00"
8	12	5'00"
9	11	1'00"
10	20*	2'00"
11	10	3'00"
12	20	5'00"
13	9	15'00"
14	8	1'00"
15	20	2'00"
16	7	0'40"
17	13	0'10"
18	20	1'15"
19	14	0'15"
20	15	1'40"
21	20	2'00"
22	16	1'30"
23	17	1'00"
24	21	4'00"
25	18	2'00"
26	20	5'00"
27	6	1'00"
28	5	2'00"
29	4	2'00"
30	3	2'00"
31	2	5'00"
32	1	5'00"
33	End	—

*Reservoirs 20 and 21 are running tap water.

Potassium permanganate working solution should be made and placed in the appropriate reservoir. Silver nitrate working solution is made last and should be mixed and placed in the appropriate reservoir **immediately before** the staining run is begun. The agitation time interval should be set for 01" (seconds) and the agitation speed should be set for 2. Slides should be coverslipped using a xylene-based permanent mounting medium.

Results

Our pathologists reported that the results of the automated staining were comparable to those of the manual staining typically performed in our lab. Automated staining appears to yield more uniform staining throughout the section while manual staining yields areas of greater contrast. Background levels were acceptable with both methods. Our pathologists felt that either method would be acceptable for the evaluation of liver disease in patient slides (Figs. 1 and 2).

Discussion

In this study we set out to automate an ammoniacal silver stain for the visualization of reticular fibers. We chose to automate this particular staining procedure because it is tedious, time consuming, and is often run in quantities that are not easily performed manually.

When automating this staining procedure we had to tackle the following major obstacles:

• Metal may not come into contact with the silver solution at any time

• The ammoniacal silver solution must be made just prior to its use during the staining procedure

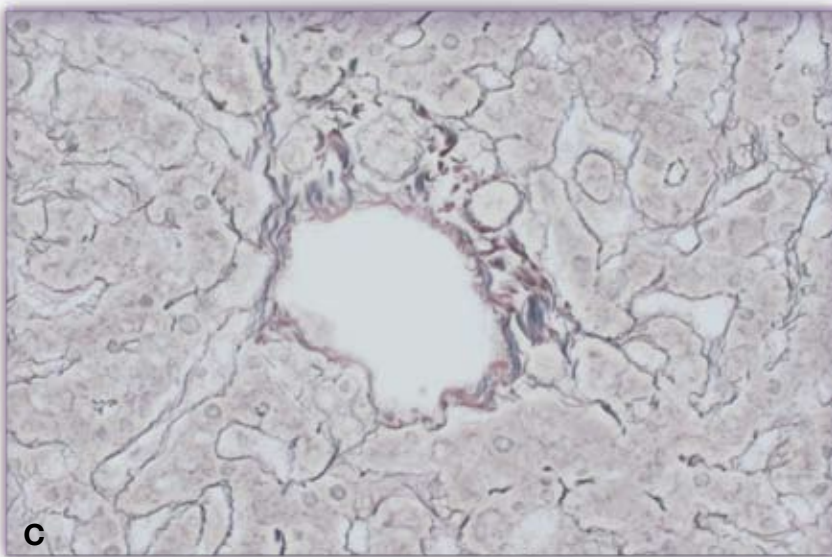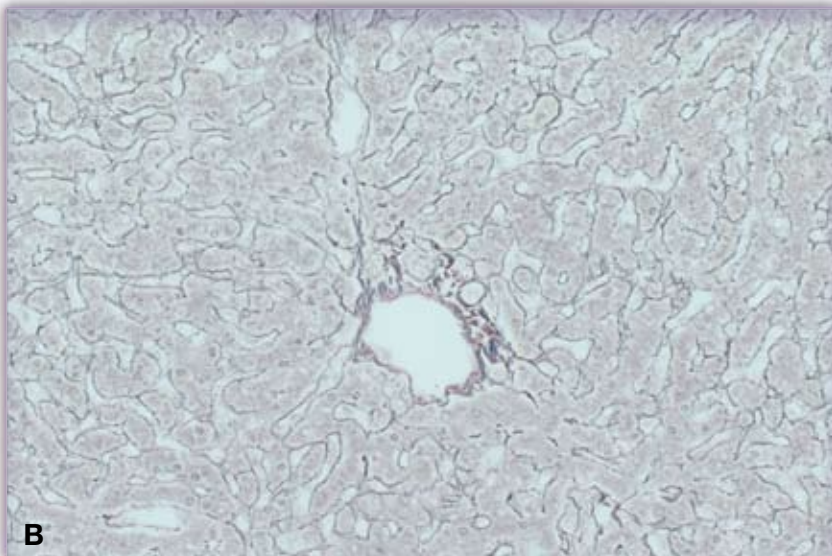• Translating "number of dips" performed by the instrument into an actual length of time in minutes

Fig. 1. Paraffin-embedded human liver, cut at 5 microns.
Gordon and Sweets stain performed manually (A) 10X; (B) 20X; (C) 40X.

We began by looking at commercially available slide baskets and adaptors that were not metal. We were able to find plastic slide baskets through Sakura Finetek, however, only metal adaptors were available to hang them on the mechanical arm of the stainer. So we began to adjust solution volumes (especially the silver nitrate working solution and formaldehyde 37%) to keep the metal hanger from touching the solutions. We determined that a volume of 360 mL is sufficient to cover a section of tissue that is placed in the center of a glass slide, and is appropriately displaced whether running 1 slide in 1 rack or 60 slides in 3 racks.

Next, we ran a stability study in which we filled all reservoirs except for the potassium permanganate working solution and the silver nitrate working solution. We determined that these solutions are sufficiently stable if prepared immediately before the stainer begins.

Lastly, we estimated the required duration for the critical dipping steps in the silver nitrate and formaldehyde by timing a technician as she was dipping the slides into these solutions when performing the stain manually. We determined that 40 seconds in the silver nitrate and 15 seconds in formaldehyde were ideal for our protocol.

While some laboratories perform a counterstain when staining reticular fibers, our laboratory prefers reticular stains without a counterstain to avoid masking the delicate reticular network commonly observed in liver sections.

Conclusions

There are few staining techniques in the histology laboratory that are as challenging to perform as the silver stain. More challenging still is to automate a staining protocol that requires precise timing of critical steps in the method. We have successfully overcome the three major obstacles of this silver stain procedure: the use of metal in a silver stain, the stability of solutions, and the conversion of the vague term "dips" into real time. In our experience, from this small sampling, the quality of results from our automated procedure equaled those from our manual procedure.

By automating this technique, we were able to minimize the run-to-run variability commonly experienced when the stain is performed manually by a variety of individuals. This staining procedure is notoriously labor-intensive, requiring approximately 1½ hours of tech time. Automating this silver stain technique for the detection of reticular fibers has enabled us to free up one of our staff to perform other laboratory duties, which in turn improves lab efficiency.

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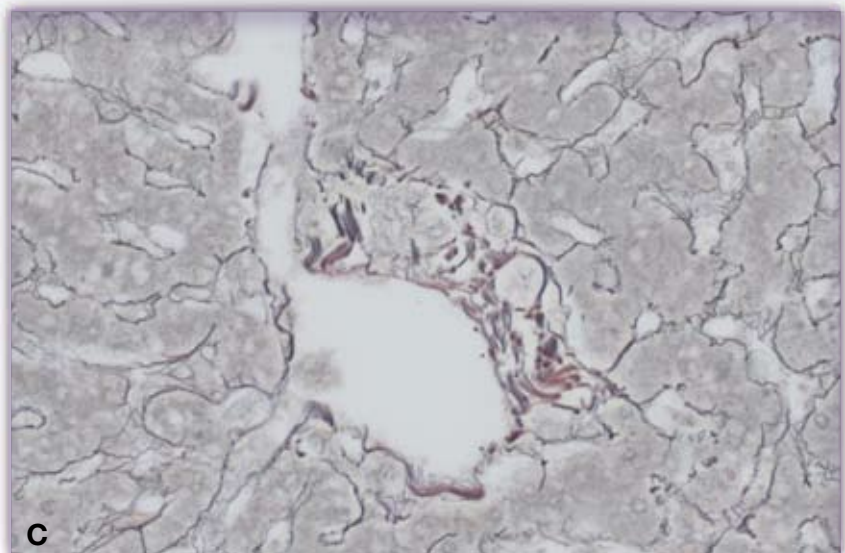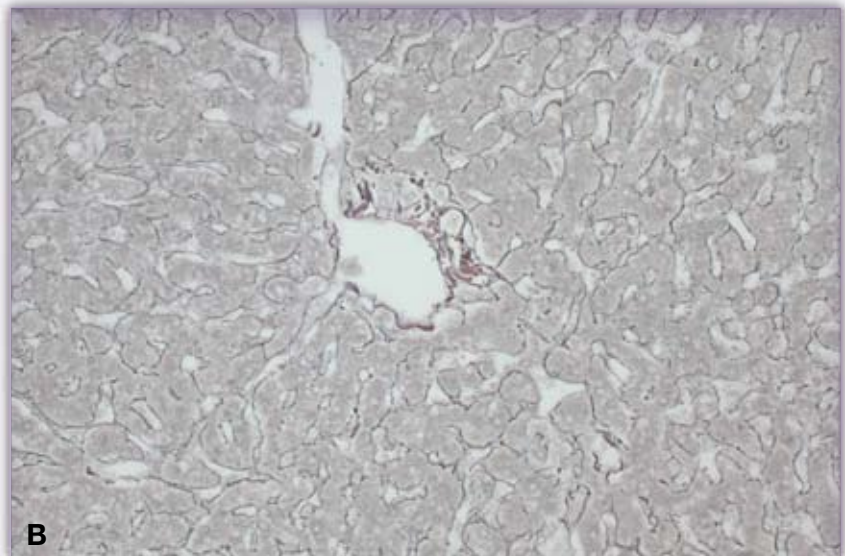
Acknowledgments

Fig. 2. Paraffin-embedded human liver, cut at 5 microns. Gordon and Sweets stain performed automatically (A) 10X; (B) 20X; (C) 40X.
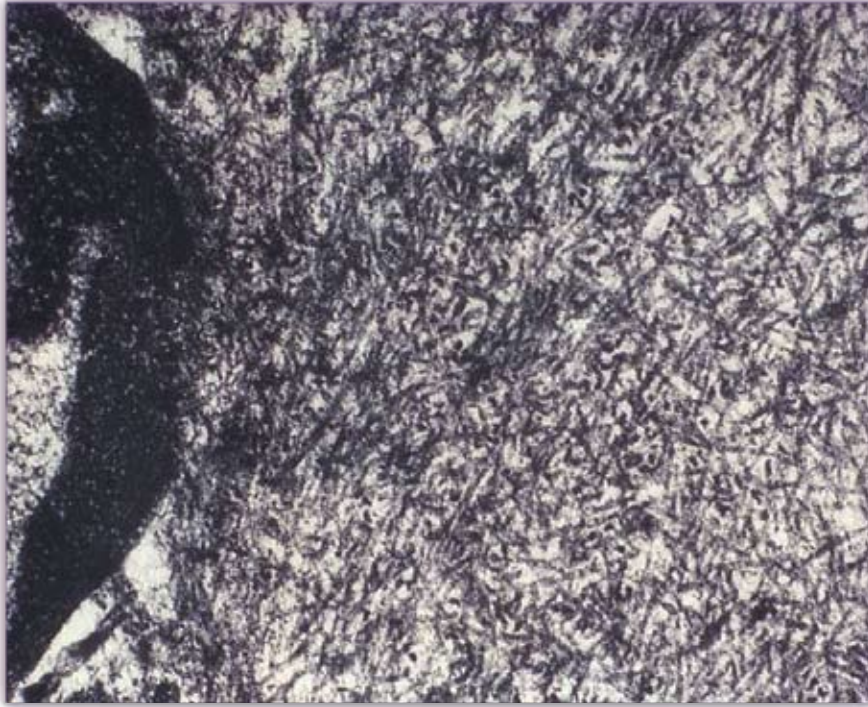
Fig. 1. Electron micrograph demonstrating the somewhat haphazard deposition of amyloid fibrils in tissue. 100,000X

A Review of Amyloid and Its Associated Pathology

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Amyloidosis is a very unique disease. The fact that at least 25 unrelated proteins with original forms very different from one another are deposited in tissue in the same structure[1] is somewhat of a phenomenon. By definition, amyloid is an amorphous, eosinophilic, misfolded protein that exhibits apple-green birefringence under polarized light when stained with Congo red. It consists of one major fibrillar protein with numerous nonfibrillar components.[2] These include amyloid pentagonal component (AP), apolipoprotein E,[2] and 1%-5% carbohydrates,[3] including proteoglycans and highly sulfated glycosaminoglycans.[4] Amyloid is soluble in water and buffers of low ionic strength[2]; it is also refractile, autofluorescent, and very weakly birefringent.[3] It is demonstrated as plaques or deposits in tissue and is usually extracellular; however, there have been cases of amyloid deposition in macrophages and plasma cells.[2]

Amyloidosis is the disease associated with amyloid deposition, although it should not be considered just one disease, as there are many different proteins that can form amyloid deposits. Amyloidosis can affect tissue and organ function in a variety of ways. The mere physical pressure of the accumulating protein may harm organs by reducing the flow of nutrients to cells.[5] Amyloid will eventually replace the parenchyma of tissue, causing loss of tissue function and, ultimately, death.[3]

Early documented cases of amyloidosis date back to the 17th century when it was referred to as "lardaceous disease" and "waxy degeneration," terms that resulted from the gross appearance of the diseased organ.[6] The term amyloid, which means starch-like, was first coined by the German botanist, Matthias Schleiden (1838); however, it was Rudolph Virchow who first applied the term to humans in 1854. The reaction of amyloid when stained with iodine led him to believe it was identical to starch while others believed it to be cellulose.[6] The discovery of its high nitrogen content implied that amyloid was neither starch nor cellulose, but a protein.[5]

The amyloid fibril was first visualized with electron microscopy (EM) by Cohens and Calkins in 1959.[6] In an electron micrograph, amyloid appears as an accumulation of haphazardly strewn fibrils, each composed of two filaments that are arranged in an antiparallel beta-pleated sheet conformation. Fibrils are 8 to 10 nanometers in width and can be up to many millimicrons in length.[3] At low magnification (5000X), fibrils cannot be resolved and look cottony in appearance, however, at high magnification (100,000X), an electron-lucent core can be seen (Fig. 1).[7]

Amyloidosis is defined as either localized or systemic, and can be classified in two ways. Until the late 1960s and early 1970s, amyloidosis had been categorized based on organ distribution and clinical findings.[2] For example, primary amyloidosis occurs spontaneously, not as a result of another disease. It usually affects muscle, heart, skin, and tongue, and the deposits are generally confined to a specific area.[3] Secondary amyloidosis arises due to another, typically chronic, illness such as tuberculosis or rheumatoid arthritis. It normally affects the liver, spleen, kidneys, and adrenal glands, and deposits are usually systemic in nature.[3] This more qualitative method of classification is no longer recommended.[2]

Today, because of the availability of biochemical and molecular techniques, amyloid may be categorized according to its derivative protein.[5] This classification nomenclature designates the letter "A" for amyloid followed by a letter describing the source protein.

There are approximately 25 source proteins currently identified that may lead to amyloid deposition, however, the underlying causative process is still not well understood.

Amyloid A protein (AA) is derived from amyloid P component that circulates bound to lipoprotein.[2] Formerly designated as secondary, it is related to chronic inflammatory diseases, microbial infections, and neoplasms.[2] Most often it affects kidney, liver, and spleen,[2] and it is the most common type of amyloidosis found in developing countries.[1] In the United States, amyloid type AL is most prevalent.[1] Amyloid light chain protein is produced by plasma cells and consists of either the entire light chain of an antibody or portion thereof.[8] The deposition of light chain lambda, rather than kappa, appears to be most prevalent.[4] It was formerly referred to as primary amyloidosis or myeloma-associated amyloidosis, as it is related to multiple myeloma.[2] Typically, most patients with AL-type do not have a classic multiple myeloma condition; however, they do have B-cell proliferation of an abnormal protein, rather than tumor production.[8] This type will usually target organs such as the heart, kidney, gastrointestinal tract, respiratory tract, and peripheral nerves.[2]

Familial transthyretin-related amyloidosis (ATTR) is derived from transthyretin, a plasma protein produced largely in the liver.[2] ATTR is the most common type of hereditary amyloidosis in the United States.[1] Normal-sequence ATTR is also known as senile cardiac amyloidosis, and mutant-sequence ATTR is the autosomal dominant inherited form, which deposits mostly in the heart, gastrointestinal tract, peripheral nerves, and vitreous.[2] Beta protein amyloid, or AB, comes from a transmembrane glycoprotein; it can be found in the core of plaques in patients with Alzheimer disease and also in the walls of their cerebral blood vessels.[2] Individuals with Down syndrome may also develop this type of amyloid, especially in their fifth decade of life.[2]

The exact cause of amyloidosis is unknown. Some types are inherited, as with ATTR; AA stems from another underlying disease. The explanation for why these proteins do not metabolize, and instead build up, remains a mystery. There are certain factors that place a person at risk for developing amyloidosis; these include advanced age, the male sex, family history, long-term kidney dialysis, and a history of other diseases.[9]

Amyloidosis is sometimes difficult to diagnose, especially because symptoms can be vague or nonexistent.[9] This is dependent on which organs are affected, but general symptoms include fatigue, weight loss, shortness of breath, diarrhea, macroglossia, changes to the skin, irregular heartbeat, and difficulty swallowing.[9]
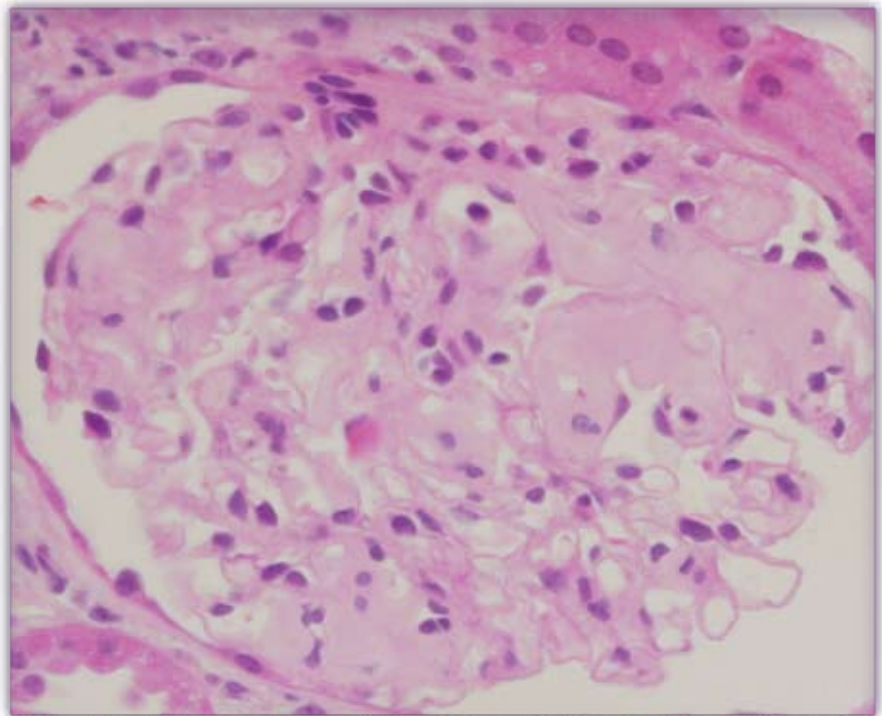

Fig. 2. Renal glomerulus stained with H&E displays pink amorphous areas that are consistent with the appearance of amyloid. 400X

If the disease is suspected, blood and urine tests may be performed to detect any abnormal protein[9]; 90% of patients with AL have monoclonal immunoglobulin light chains in their blood or urine.[7] Definitive diagnosis, however, requires a biopsy. The biopsy site can be the affected organ, although there are situations where this is not appropriate, as for example when the brain is involved.[3] Optimal biopsy sites are bone marrow, kidney, and rectum,[3] and subcutaneous fat aspirations are also common.[2] The latter, less invasive procedure can replace the need to biopsy a major organ, as amyloid is often present in subcutaneous fat capillaries in patients with systemic amyloidosis.[2] Bancroft[3] claims that amyloid can be found in 90% of rectal and/or subcutaneous fat biopsies in patients with systemic amyloidosis.

Grossly, organs affected by amyloidosis look enlarged, firm, pale, and waxy. Histologically, amyloid has a glassy, cartilage-like appearance, and it stains pink with hematoxylin and eosin (H&E) stain (Fig. 2).[10] It has been proposed that frozen sections are optimal for demonstrating amyloid,[3] although this is usually impractical in a clinical setting. After biopsy, the tissue is sent to the laboratory where it is to be processed. A portion of each biopsy may be set aside for electron microscopy.

Choice of fixative for routine histology is not critical. Formalin is suitable, although staining may be less intense with prolonged formalin storage,[3] and Carson[11] prefers alcohol fixatives.

Control tissue should be as fresh as possible as it tends to lose reactivity if stored too long.[3] Generally, smaller deposits of amyloid will stain more strongly than larger, presumably older deposits.[3]

As mentioned earlier, the first stain used to demonstrate amyloid was iodine. During the late 1800s, metachromatic stains were found to stain amyloid better than iodine,[3] but the dye that replaced them both and still remains the gold standard for amyloid demonstration is Congo red. Congo red is an aniline dye first used in the textile industry.[3] It was utilized beginning in 1886 to stain tissue, but it was not used for amyloid staining until Bennhold's technique in 1922.[3] Seven years later, two Belgian pathologists were the first to document that Congo red stained amyloid (Fig. 3), exhibiting an apple-green birefringence with polarized light (Fig. 4).[6]

Even with the availability of immunohistochemical stains, Congo red with apple-green birefringence remains the definitive diagnostic criteria for amyloidosis today. The stain will also emit red with fluorescence microscopy as Congo red is a fluorescent dye[10]; however, this should not be considered as specific for amyloid (Fig. 5).[3]

The mechanism of staining was unknown for a long time but it is now believed to be accomplished through hydrogen bonding.[3] Dye molecules disturb the hydrogen bonds that hold the beta-pleated sheets of the fibrils together and then the dye molecules insert themselves between the sheets, reestablishing new hydrogen bonds.[3] These new bonds uphold the stability and shape of the amyloid. Integrity of the beta-pleated sheet configuration and dye molecules must be maintained in order for the Congo red stain to be successful (Fig. 6). According to Carson,[11] it is unknown whether the hydrogen bonding occurs with the protein or carbohydrate component of the fibril. Although Congo red binds many tissue components, its greatest attraction is for amyloid.[10]

There are many different Congo red staining techniques, including those by Puchtler and Highman. Because Congo red has electrostatic attractions to other tissue components,[3] these methods
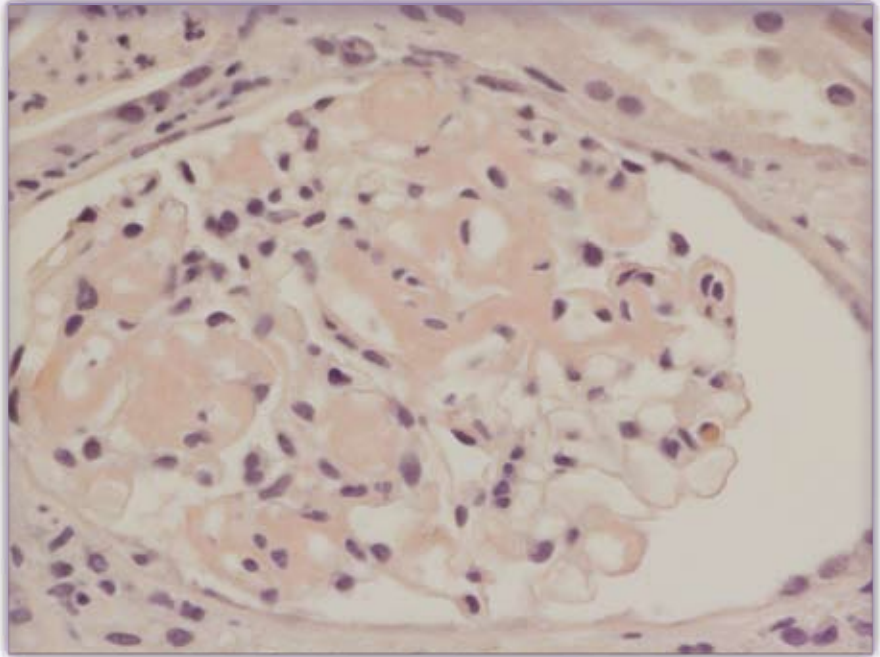

Fig. 3. The same renal glomerulus (as in Fig. 2) stained with Congo red displays areas of pink to light red that are consistent with the appearance of amyloid. 400X
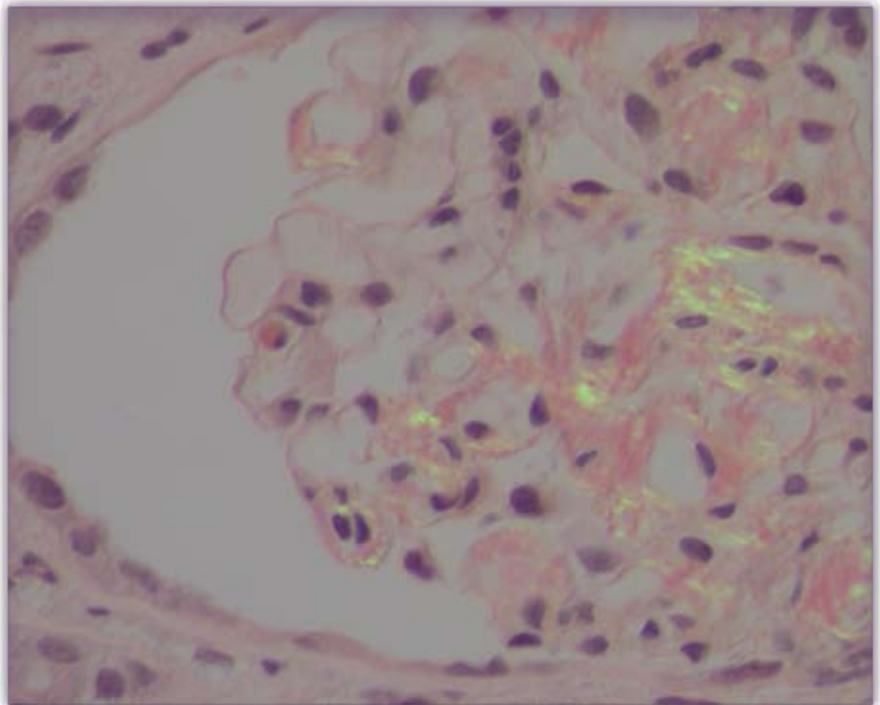

Fig. 4. The renal glomerulus stained with Congo red from Fig. 3 exhibits the characteristic apple-green birefringence associated with amyloid deposition when seen with polarized light. 400X

take precautions to inhibit or lessen nonspecific background staining. Carson[11] declares Puchtler's method to be the method of choice.

It utilizes an alkaline alcoholic sodium chloride solution prior to application of the dye. The salt and alcohol help to neutralize charges in the tissue, thus keeping background staining to a minimum.[11] Salt is used in the actual Congo red stain solution for the same purpose.[10] The solution also helps to enhance the hydrogen bonding of the dye to the amyloid fibril.[3] The method by Highman incorporates an alkaline salt solution after the dye, so as to differentiate the tissue.
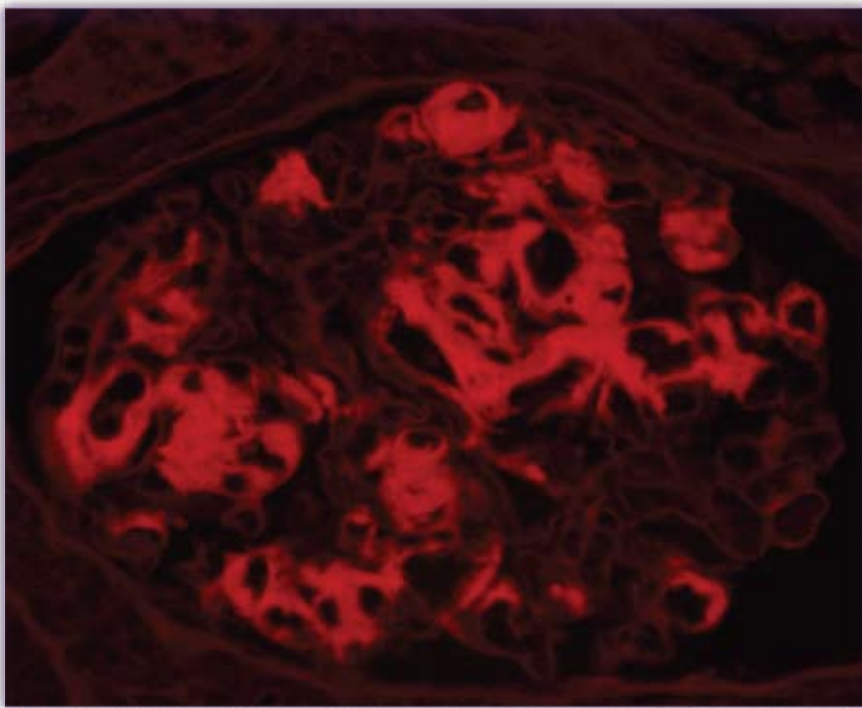

Fig. 5. The same renal glomerulus stained with Congo red as in Fig. 3 when viewed with fluorescence microscopy demonstrates vivid red staining of amyloid deposits. Excitation wavelength 546 nm (BP 546/12 excitation filter); 400X

There are some things to keep in mind while performing a Congo red stain for detecting amyloid. Tissue sections must be cut between 8 and 10 microns; sections cut too thin will yield red birefringence, and sections cut too thick will appear yellow under the polarizing microscope.[3] Working solutions must always be made just prior to use because of their limited stability.[10] Although steps are taken to avoid background staining, collagen, keratin,

and elastin fibers may also bind the dye; however, apple-green birefringence of the amyloid will be the differentiating factor.[10] There are other structures that exhibit apple-green birefringence with Congo red because they have the same beta-pleated sheet configuration. Cellulose and chitin, for example, can be ruled out on morphological grounds.[3] Congo red is the most widely used stain for amyloid, although, there are a number of other stains that have been used as well. Sirius red, another textile dye, has been used to stain for amyloid and is believed to provide more intense staining than Congo red: it has not, however, caught on in popularity,

probably because it does not have the birefringent property of Congo red.[3]

Thioflavine T is a basic fluorochrome dye that emits yellow fluorescence and was introduced in the late 1950s to stain amyloid.[3] It is not as specific as once believed, but some prefer it due to the greater sensitivity of the fluorescence technique, which allows very small deposits of amyloid to be visualized.[10]

Crystal violet is a metachromatic dye that has had some popularity for staining amyloid. Although not considered specific for amyloid, this stain is simple and can be used as a quick screening method.[11] The actual mechanism of staining is not known, although it is thought that the metachromasia is due to the carbohydrate component of amyloid.[11] On the other hand, crystal violet is a mixture of three basic dyes[3]; amyloid may simply react with one of them, suggesting a polychromatic reaction.[11]

Amyloid will bind many other stains. Both alcian blue and toluidine blue react with the carbohydrate component of amyloid, although results are not optimal with either dye.[3] With toluidine blue, amyloid exhibits red birefringence, but small deposits may be difficult to distinguish.[10] Amyloid is variably periodic acid-Schiff positive; it appears green or blue with a trichrome stain, and it stains a khaki color with the van Gieson stain.[3] Silver impregnation methods are also useful in identifying amyloid plaques in the brains of patients with Alzheimer disease.[3]

"After histologic diagnosis of amyloid, it is mandatory to classify the amyloid protein(s) to assess patient prognosis and treatment…." [12] While Congo red and other special stains can identify the presence of amyloid, immunohistochemical methods are employed to determine the specific source protein. For example, in patients with cardiac amyloidosis, both AL and ATTR can present similarly in the clinical setting.[2] Because these types of amyloid require different treatments, differentiation is essential.[3] A general immunohistochemical stain that can be performed on all amyloid is the anti-amyloid P component. It should be noted that this is a general stain, and will not differentiate types of protein. Also, AP is identical to serum amyloid P component, a circulating plasma protein found in basement membrane and elastin fibrils. Nonspecific staining and/or false-positive staining may occur for this reason. Anti-amyloid P component should only be used along with Congo red.[3]

Immunohistochemistry for amyloid did not become popular at the outset in the 1970s, probably because results can
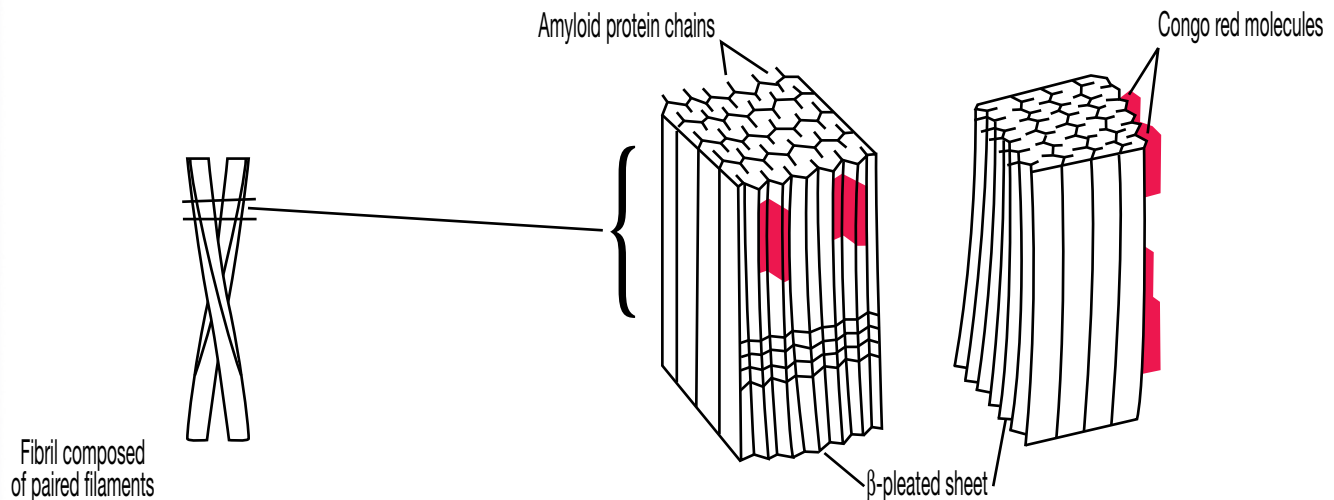
Fig. 6. Despite the haphazard manner in which amyloid fibrils are deposited in tissue (as seen in Fig.1), the structure of each fibril is highly organized as shown in this illustration.

be extremely variable. Epitopes may be masked due to the protein's beta-pleated sheet configuration, and even epitope retrieval may be unsuccessful.[3]

AL results are particularly variable; this is because many commercial antibodies are raised against the constant region of the light chain.[1] In some cases of AL, the amyloid fibrils are composed of the variable region only with no portion of the constant region, so no binding will occur.[1]

Due to the high variability in immunohistochemical results, it is very important to run a complete titer of all epitope retrieval methods to determine what is optimal for each antibody. Bancroft has found that concentrated formic acid was a successful retrieval method for both AA and ATTR. It is also wise to run a panel of antibodies. Antibodies are available against most types of amyloid, although typically only specialized amyloid centers keep antibodies against the rarer forms. It is useful to keep the more common types, such as AA and AL, on hand for typing.[3]

There is no cure for amyloidosis, but there are treatments to help control symptoms and limit the production of more amyloid deposits.[9] Prognosis and treatment depend on the specific

type of amyloid, but most types can be managed with medication and diet.[9] Early detection is best, and in some cases, long-term survival is possible.

Those who suffer from light chain amyloidosis (AL) and do not receive treatment usually do not survive more than 1 year after diagnosis; however, with treatment, they may survive up to 4½ years.[13] Patients with hereditary types of amyloidosis, including ATTR, may survive up to 15 years after the disease surfaces, and depending on how well the primary condition is treated, AA patients can live for more than 10 years.[14]

In summary, amyloidosis can be divided into many different diseases, all based on the amyloid's source protein. It is quite a mysterious disease, and more causative proteins continue to be discovered. By identifying the type of amyloid through a biopsy and clinical tests, patients will be able to receive the optimal treatment, which can help control symptoms and even prolong life.

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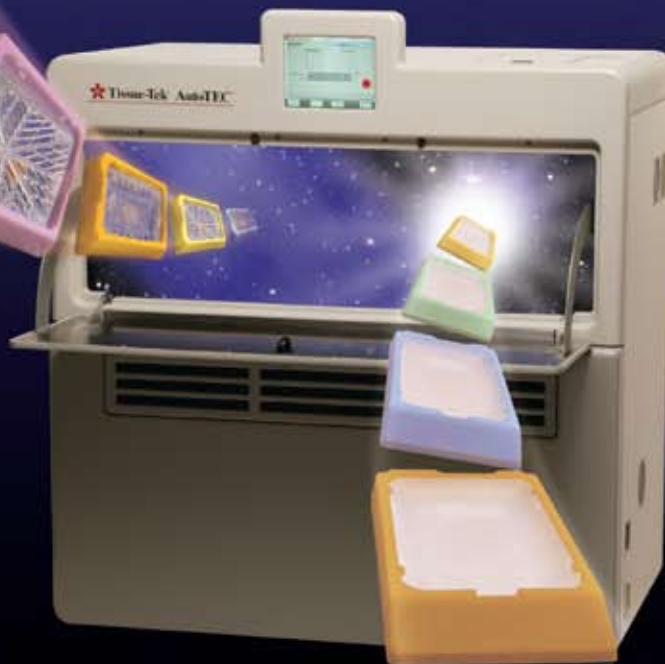
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Educational Opportunities in 2008

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

6-8   **NSH Region VII Symposium/Convention**
Title: **A Great Team: Supply and Demand**
Site: Grace Inn
   Phoenix, AZ
   www.graceinn.com
Contact: Arizona Society for Histotechnology
   www.azhistology.net

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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