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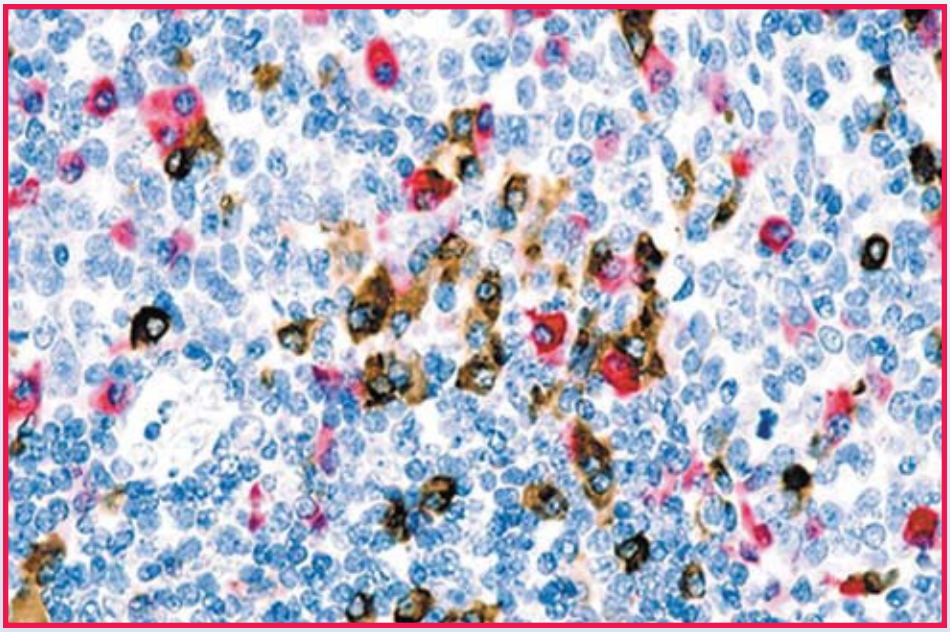

Fig. 1. Kappa-lambda double-stain: simultaneous staining of kappa and lambda by diaminobenzidine (DAB) and fast red (FR), respectively (with hematoxylin counterstain). Brown cells are kappa-positive; red cells are lambda-positive. Axillary lymph node, 40X

The Technical, Clinical, and Financial Benefits of Multiantigen Immunostaining (MAIS) Procedures

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Introduction

Multiantigen immunostaining (MAIS) represents a specialized form of immunohistochemical (IHC) staining that allows two or more antigens to be localized by immunologic methods and microscopically differentiated from one another on the basis of morphology (cellular location) and/or color. MAIS has become increasingly more popular in recent years as pathologists and other scientists begin to appreciate the significant technical, clinical, and financial benefits that such procedures provide, including:

- Improved correlation of the immunostaining patterns of different antigens within adjacent cells and tissues, rather than having to interpret results from two or more individually prepared slides — as is the case in most diagnostic and research situations
- Reduction in the number of slides that need to be examined in order to properly evaluate many clinical cases or validate research experiments, thereby decreasing the time necessary to make a diagnosis or collect essential data
- Conservation of specimen material — which may be critical when only a small amount is collected originally or is available (eg, prostate core biopsies)
- Reduction in the number of slides that are prepared and stained by immunologic methods
- Reduction in the reagent volumes that are consumed in the process of deparaffinizing, hydrating, immunostaining, counterstaining, dehydrating, clearing, and coverslipping, with a corresponding reduction in the amount of chemical waste that is generated
- Reduction in the number of slides that must be labeled, handled, and stored, with a corresponding reduction in physical labor required to perform these processes and the potential for errors

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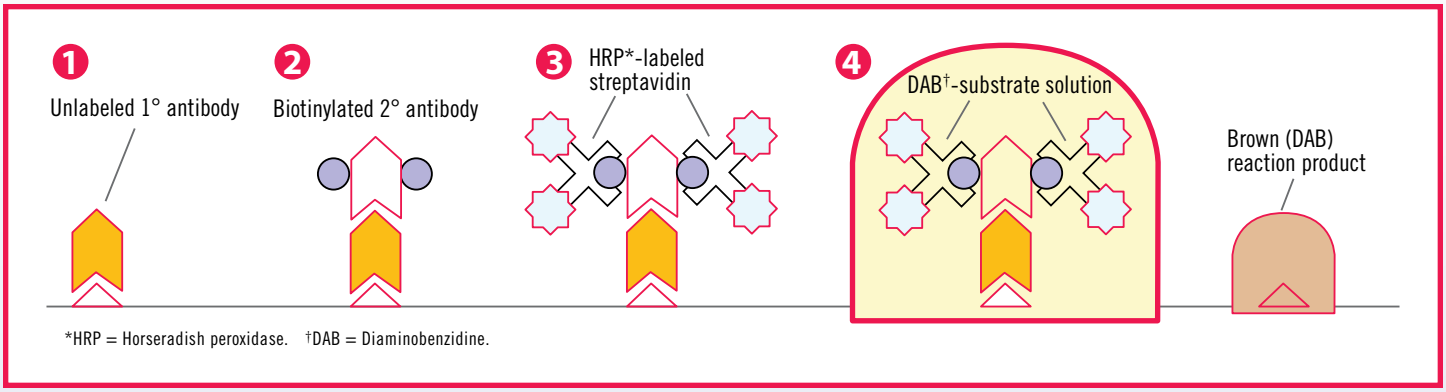

Fig. 2. Localization of one antigen using a monoclonal or polyclonal antibody: 4 steps.

Technical Considerations

There are basically two types of MAIS protocols: **sequential**, where one IHC procedure is performed immediately after another, and **simultaneous**, where two different antigens are localized through one IHC procedure. One of the best ways to demonstrate how MAIS procedures work is to compare them to standard IHC staining reactions (Fig. 2), which usually involve four distinct reagent applications separated by buffer rinses: 1) an unlabeled primary antibody, 2) a biotinylated secondary antibody, 3) an avidin-enzyme conjugate, and 4) a substrate-chromogen solution.

Sequential MAIS procedures were the first to be developed, and continue to be used today. They are particularly effective when the primary antibodies used to localize target antigens are *produced in the same animal species,* or when the same enzyme label is used with different chromogens. In this case, one complete 4-step procedure is performed, a denaturing solution is applied, and then another 4-step procedure is performed (Fig. 3). This approach to MAIS is advocated by IHC product vendors such as Dako and Ventana, whose antibodies and biotin-streptavidin detection reagents can be used to perform MAIS either manually or as part of an automated system.

In contrast, *simultaneous* MAIS procedures (Figs. 4, 5) use two or more primary antibodies that are *produced in different animal species,* and permit localization of as many as four antigens in two different colors within procedures that involve the same number of steps as standard single-antigen IHC protocols. In this case, a cocktail of monoclonal and

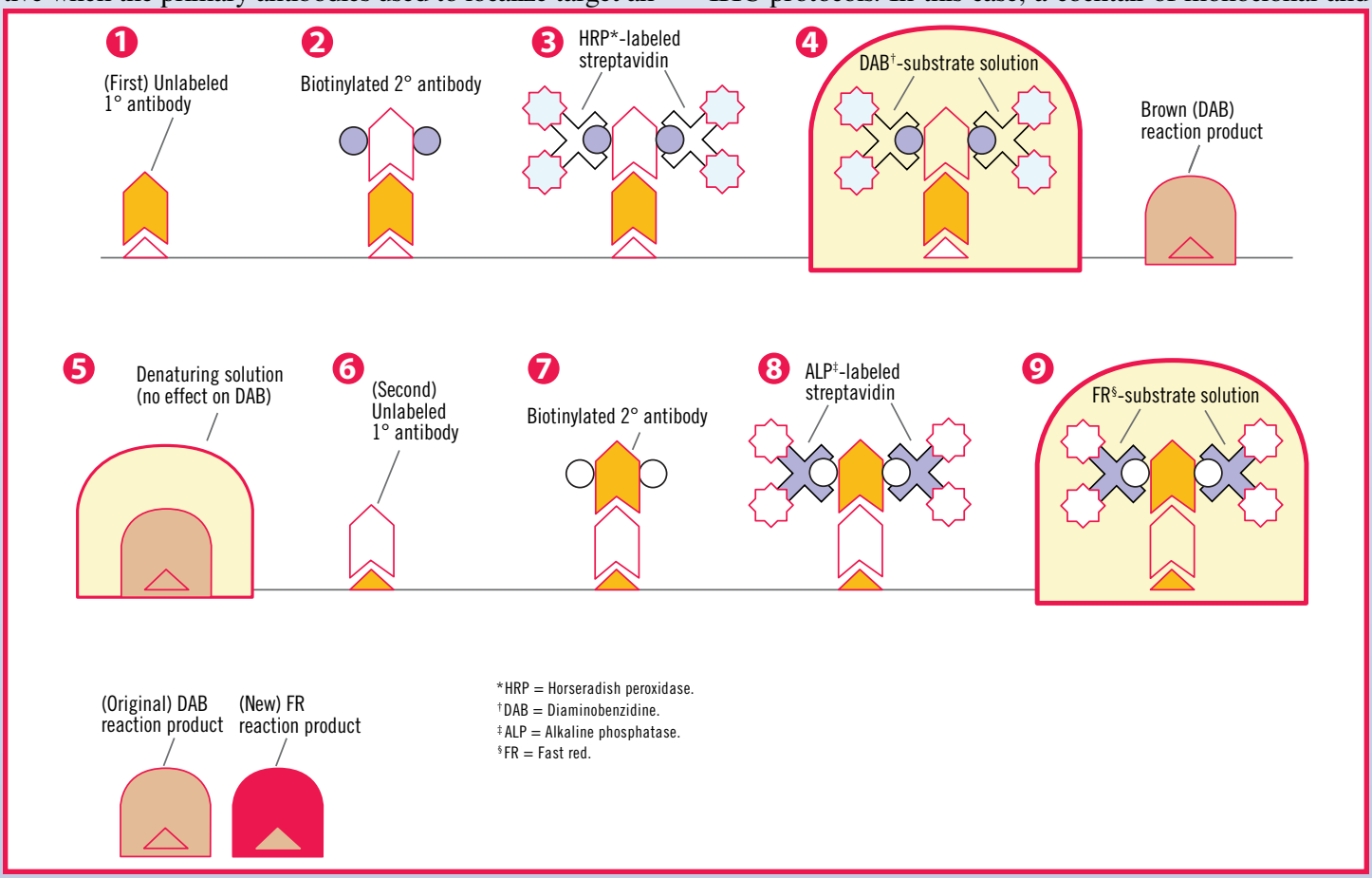

Fig. 3. Localization of two antigens using two antibodies in a sequential procedure: 9 steps.

polyclonal primary antibodies (directed at different antigens) is applied as a single reagent, followed by a cocktailed detection reagent (eg, horseradish peroxidase-labeled anti-mouse and alkaline phosphatase-labeled anti-rabbit), and two separate substrate-chromogen reactions (Fig. 4). This approach to MAIS is promoted by Biocare Medical, who also offers a wide variety of prediluted primary antibody cocktails (Table 1) and detection reagents, including chromogens in five different colors.

In addition to the advantages described above, other technical considerations of *simultaneous* MAIS include:

- The opportunity to perform only one pretreatment — eg, enzyme digestion or heat retrieval[1] procedure — if necessary
- Use of polymer detection systems, which eliminate the nonspecific staining that is observed when biotin-streptavidin detection reagents are used on specimen material containing a significant amount of endogenous biotin
- The need to use Tris-buffered saline (TBS)-based rinse solutions, since the alkaline phosphatase label used within most MAIS procedures does not react well in wash buffers composed of phosphate-buffered saline (PBS)

Clinical Considerations

Although there is almost no limitation to the variety of antigen targets that could be studied simultaneously using MAIS techniques, there are some antibody combinations that are considered more useful than others (Table 1). Some of the most useful MAIS protocols that have come into common use include the kappa and lambda double-stain (Fig. 1) and the PIN-4 triple-stain (Fig. 6), which is used for diagnostic and prognostic purposes in cases of suspected prostate cancer. Due to space constraints, it is not practical to provide photomicrographs of the multitude of MAIS procedures that have been developed.

Financial Considerations

In addition to being clinically useful and easy to perform, MAIS is also quite cost effective.[2,3] The best way to dem-

Table 1 - Commercially Available Antibody Cocktails for Multiantigen Immunostaining (MAIS) Applications

Antigens	Cell/Tumor Types	Clinical Utility	Color
Apoptotic CK18	Apoptotic-adenocarcinoma - Cytoplasmic	Evaluation of cell proliferation (Ki-67) and cell death (Apoptotic-CK18)	Red
Ki-67	Proliferative cells - Nuclear		Brown
CD3	Endothelial cells - Cytoplasmic	Evaluation of cell death (Caspase-3) in T-cell lymphomas (CD3)	Brown
Caspase-3	Proliferative cells - Cytoplasmic		Red
CD31	Endothelial cells - Cytoplasmic	Evaluation of cell proliferation in CD31-positive tumors	Red
Ki-67	Proliferative cells - Cytoplasmic		Brown
CD34	Endothelial cells - Nuclear	Differentiation between dermatofibroma (Factor XIIIa) and dermatofibrosarcoma (CD34)	Brown
Factor XIIIa	Dermal dendrocytes - Cytoplasmic		Red
CDX-2	Colon tumor cells - Nuclear	Differentiation between colon, breast, and lung carcinoma	Brown
CK7	Breast/lung tumor cells - Cytoplasmic		Red
CK5/6	Mesothelioma - Cytoplasmic	Identification of mesothelioma (positive for both antigens)	Brown
Calretinen	Mesothelioma - Nuclear/Cytoplasmic		Red
CK5	Myoepithelial cells - Cytoplasmic	Differentiation between invasive and non-invasive breast carcinoma, and interpretation of possible crush artifact (ie, usual type vs atypical hyperplasia)	Brown
p63	Myoepithelial cells - Nuclear		Brown
CK8/18	Ductal cells - Cytoplasmic		Red
D2-40	Lymphatic endothelium - Cytoplasmic	Identification of presence and/or absence of lymphatic and vascular endothelium within tumors	Brown
CD34	Vascular endothelium - Cytoplasmic		Red
HMW-CK	Squamous carcinoma - Cytoplasmic	Differentiation between squamous cell carcinomas and adenocarcinomas	Brown
LMW-CK	Adenocarcinoma - Cytoplasmic		Red
Kappa	Kappa-positive cells - Cytoplasmic	Identification of monoclonality in lymphomas, myelomas, and plasmacytomas	Brown
Lambda	Lambda-positive cells - Cytoplasmic		Red
Ki-67	Proliferative cells - Nuclear	Evaluation of cell proliferation (Ki-67) and cell death (Caspase-3)	Brown
Caspase-3	Apoptotic cells - Cytoplasmic		Red
Ki-67	Proliferative cells - Nuclear	Evaluation of cell proliferation (Ki-67) in kappa-positive tumors	Brown
Kappa	Kappa-positive cells - Cytoplasmic		Red
Ki-67	Proliferative cells - Nuclear	Evaluation of cell proliferation (Ki-67) in lambda-positive tumors	Brown
Lambda	Lambda-positive cells - Cytoplasmic		Red
CD20	B-lymphocytes - Cytoplasmic	Differentiation between B-cell (CD20) and T-cell (CD3) lymphomas	Red
CD3	T-lymphocytes - Cytoplasmic		Brown
CD20	B-lymphocytes - Cytoplasmic	Evaluation of cell proliferation (Ki-67) in B-cell lymphomas (CD20)	Brown
Ki-67	Proliferative cells - Nuclear		Red
LCA (CD45)	B- and T-lymphocytes - Cytoplasmic	Differentiation between lymphoma and malignant melanoma	Brown
S100	Malignant melanoma - Nuclear/Cytoplasmic		Red
Tyrosinase	Malignant melanoma - Cytoplasmic	Evaluation of suspected malignant melanomas	Brown
MART-1	Malignant melanoma - Cytoplasmic		Brown
S100	Malignant melanoma - Nuclear/Cytoplasmic		Red
P504S	High-grade PIN and/or carcinoma - Nuclear	Differentiation between benign prostate lesions, high-grade PIN, and invasive carcinomas	Red
HMW-CK	Basal cells - Cytoplasmic		Brown
p63	Basal cells - Nuclear		Brown

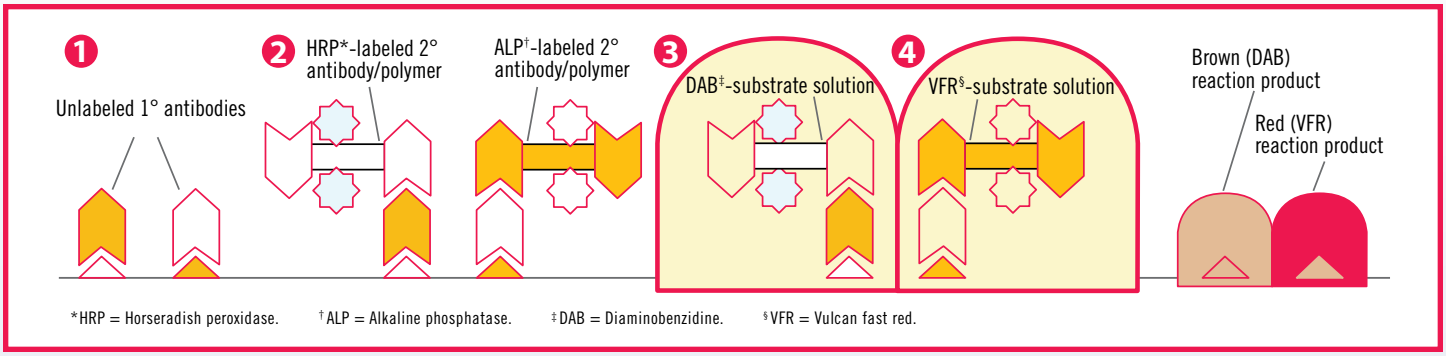

Fig. 4. Localization of two antigens using cocktailed (monoclonal/polyclonal) antibodies and cocktailed (HRP/ALP) detection reagents: 4 steps.

onstrate this is by comparing the cost of the reagents required to perform three separate IHC procedures on three slides to the cost of reagents used to produce three stains on the same slide (Table 2), as is the case with the PIN-4 stain. This financial advantage is particularly important in laboratories that obtain prospective payment for their IHC staining services, since a reduction in the cost of materials (as is achieved in nearly all MAIS procedures) will directly impact the laboratory's net revenue. Without going into detail on the billing and reimbursement mechanisms employed within the clinical laboratory industry, suffice it to say that, as a general rule, a laboratory seeks payment for each and every slide that is stained and interpreted in order to produce a diagnosis in a given case. It is important to point out, then, that when MAIS procedures are properly executed, a laboratory can seek payment for each antigen that is identified on a single slide, rather than having to produce a separate slide for each IHC stain.

Although the author is not aware of any government regulations or accrediting agency standards that specifically address use of MAIS procedures, the most important issues to consider are *how one defines and uses an antibody cocktail,* and *how the resulting specimens are interpreted.* One type of cocktail consists of a mixture of antibodies to two (or more) epitopes of the same or similar antigens (often produced in the **same** species), that is *designed to improve the localization of a single cell/tissue target.*

The other type of cocktail is a mixture of antibodies to different antigens, usually produced in **different** species, that is *designed to identify different antigens within different cells.* Therefore, if application of an antibody cocktail is *intended to localize a single target-antigen, and the additional localization* attributable to the added antibodies *cannot be*

Table 2 – Reagent Cost Comparison Between Single-antigen and Triple-antigen IHC Procedures

SINGLE-ANTIGEN STAINING

INDIVIDUAL ANTIBODIES

Reagent Description	List Price per Unit	Cost per Slide
P504S - Polyclonal [P504S], 6 ml, 40 slides	$221.00	$5.53
Cytokeratin, HMW - Monoclonal [DE-SQ], 6 ml, 40 slides	$180.00	$4.50
p63 - Monoclonal [BC4A4], 6 ml, 40 slides	$167.00	$4.18
Total cost per slide, three separate antibodies:		**$14.21**

INDIVIDUAL DETECTION REAGENTS

Mach 2-Rabbit Polymer-ALP Detection Kit, 100 ml, 660 slides	$859.00	$1.30
Mach 2-Mouse Polymer-HRP Detection Kit, 100 ml, 660 slides	$809.00	$1.23
Vulcan Fast Red (VFR) Chromogen/Naphthol Phosphate Substrate Kit, 100 ml, 660 slides	$129.00	$0.20
Cardassian DAB Chromogen/H_2O_2-Substrate Kit, 110 ml, 730 slides	$108.00	$0.15
CAT Hematoxylin, 500 ml, 3300 slides	$34.00	$0.01
Avg. cost per slide, detection of one antigen:		$2.89
Avg. cost per slide, detection of three antigens:		**$8.67**
Total cost per slide, staining of three antigens on separate slides:		**$22.88**

TRIPLE-ANTIGEN STAINING

TRIPLE-ANTIBODY COCKTAIL

Reagent Description	List Price per Unit	Cost per Slide
PIN-4 Cocktail (P504S + HMW-CK + p63), 6 ml, 40 slides	$322.00	$8.05
Total cost per slide, three-antibody cocktail:		**$8.05**

COCKTAILED DETECTION REAGENTS

Double-Stain Polymer- HRP/ALP Detection Kit 2, 100 ml, 660 slides	$1558.00	$2.36
Cardassian DAB Chromogen/H_2O_2-Substrate Kit, 110 ml, 730 slides	$108.00	$0.15
Vulcan Fast Red (VFR) Chromogen/Naphthol Phosphate Substrate Kit, 100 ml, 660 slides	$129.00	$0.20
CAT Hematoxylin, 500 ml, 3300 slides	$34.00	$0.01
Total cost per slide, detection of three antigens:		**$2.72**
Total cost per slide, staining of three antigens on the same slide:		**$10.76**

Triple-antigen staining on the same slide costs 53% less than staining for three antigens on separate slides

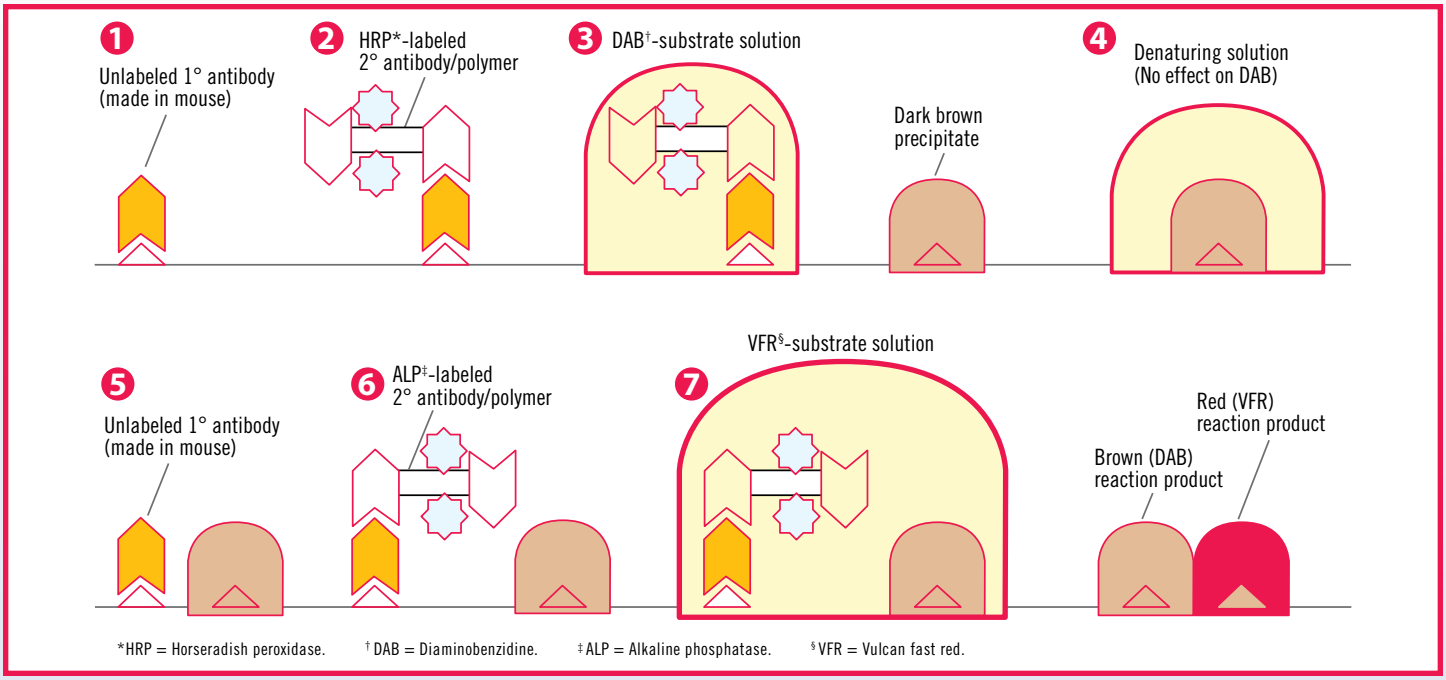

Fig. 5. Localization of two antigens using two antibodies in a simultaneous procedure: 7 steps.

microscopically differentiated from the staining that results from a single-antibody procedure (ie, the staining is the same color, or is found in the same cellular location), then the lab can charge for only one IHC procedure. On the other hand, if the antibody cocktail is *intended to localize different antigens, and these antigens can be microscopically differentiated* (ie, appear in both the nucleus and the cytoplasm of different cells, or are stained with different colored chromogens), then the lab can charge for two (or more) IHC procedures.

Performing MAIS on Automated Slide Staining Systems

Despite the fact that (simultaneous) MAIS involves the same number of steps as standard IHC staining methods, these procedures cannot be performed on all automated IHC slide staining systems. This limitation is not a function of the reagents used within the staining protocols, but, rather, of the hardware (ie, staining platform) and software (ie, operator-instrument interface) employed by these instruments.[4] In most cases, the primary shortcoming lies in the inability of these systems to accommodate reagents obtained from other vendors (eg, polymer detection-reagent cocktails), or to apply two or more substrate-chromogen solutions. It should be noted that, although some users of these restrictive instruments have been able to produce acceptable MAIS results by performing certain steps within the instrument and other steps manually, such an approach may be unnecessarily cumbersome and not cost effective.

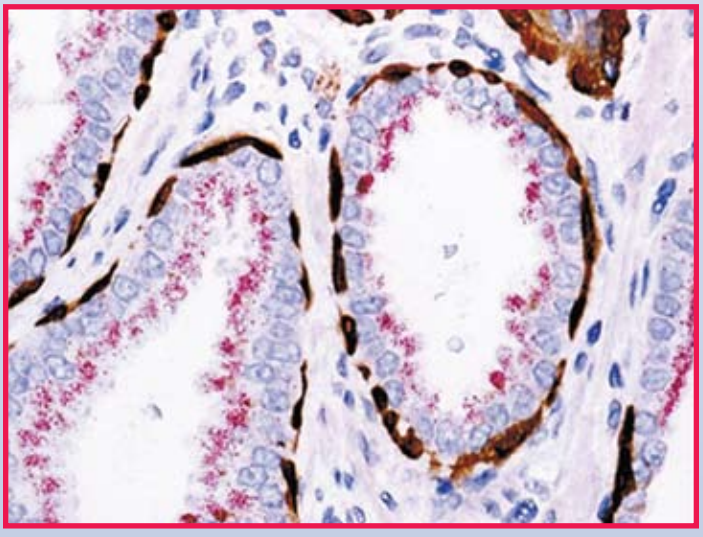

Fig. 6. PIN-4 triple-stain: simultaneous staining of HMW-CK and p63 by VFR, respectively (with hematoxylin counterstain). Brown nuclear stain represents p63 in basal cells, while brown cytoplasmic stain represents HMW-keratin on mature epithelial cells; red cytoplasmic stain represents P504S in prostate cancer cells. 40X

Conclusion

It is the author's belief that MAIS procedures, which offer improved results in a cost-effective manner, represent one of the most important technological advances in diagnostic pathology and basic science in recent years. As such, MAIS is likely to be adopted by many more laboratories as pathologists and other scientists embrace the significant technical, clinical, and financial advantages that these procedures provide. If your laboratory is not already performing MAIS, you may want to consider this approach as a means of producing enhanced results while saving time and money.

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Pigments and Minerals

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Pigments can be found in tissues in normal and pathological conditions. Because of the large variety of pigments, we will primarily consider only the ones most frequently encountered in routine histology.

Classification of Pigments

1. ***Artefact pigments:*** pigments that are not present in tissue but result from fixation and handling.

2. ***Hematogenous pigments:*** pigments that are derived from the coloring matter of the blood. They are formed by a breakdown of hemoglobin.

3. ***Autogenous pigments:*** pigments that are made by cells within the body.

4. ***Exogenous pigments:*** pigments that come from various substances outside the body and are deposited as pigments.

Artefact Pigments

Formalin pigment or *acid formaldehyde hematein* may be present in blood-containing tissue, especially if fixation is delayed or if non-buffered formalin is used for fixation. It appears as a brown-black deposit, and the recommended method for removal is to place the tissue in 70% alcohol that contains 10% ammonium hydroxide (28%) for 5 to 15 minutes.

Mercury bichloride, another artefact pigment, is present in tissues that are fixed with fixatives containing mercury bichloride such as Zenker's. It appears as a brown-black pigment and can be removed by treatment with Lugol's iodine and sodium thiosulfate (hypo).

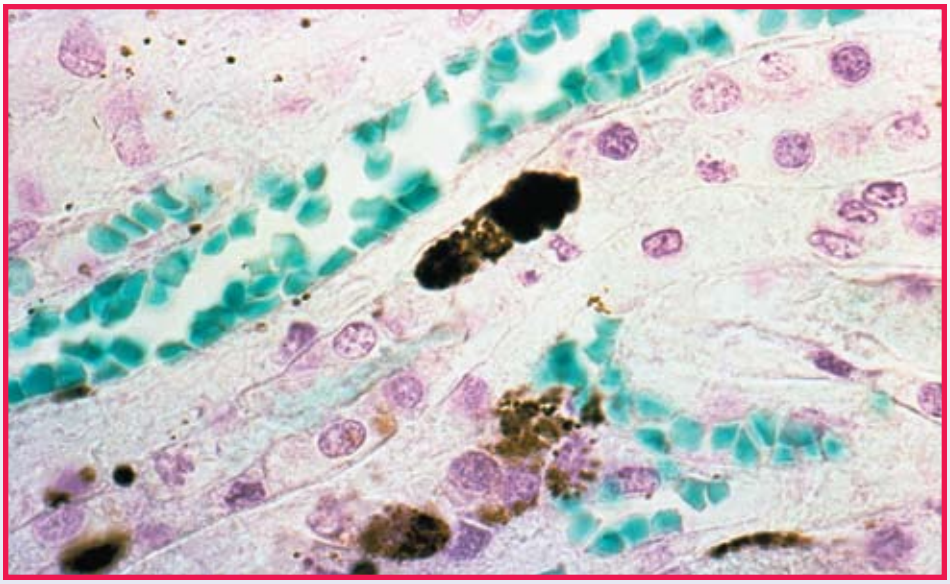


Fig. 1. A section of kidney from a patient with hemoglobinuria stained for hemoglobin with Lison's method for hemoglobin. Hemoglobin is stained blue. The black-stained material is formalin pigment. 400X

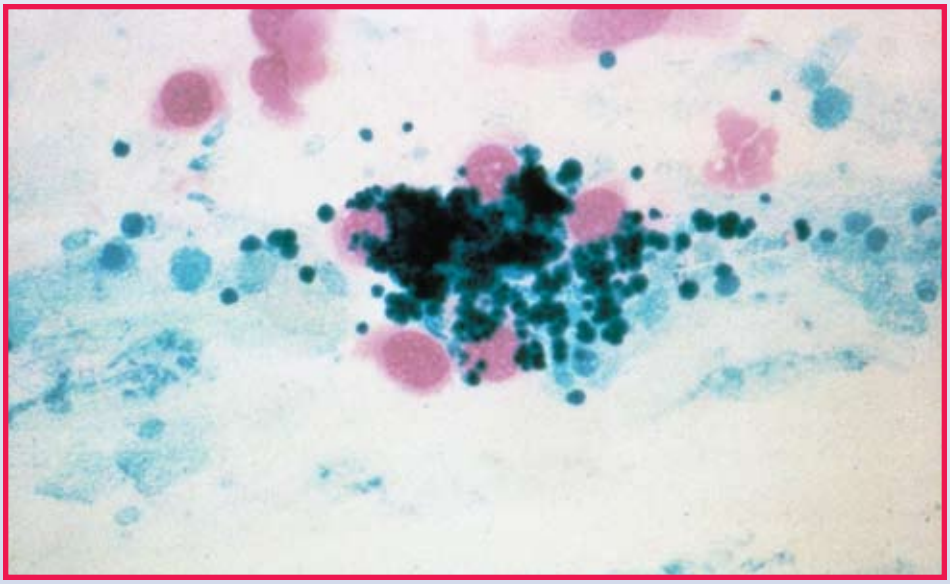


Fig. 2. A bronchial alveolar lavage stained using Pearl's method for ferric iron. Ferric iron stains blue and is known as Prussian blue. 400X

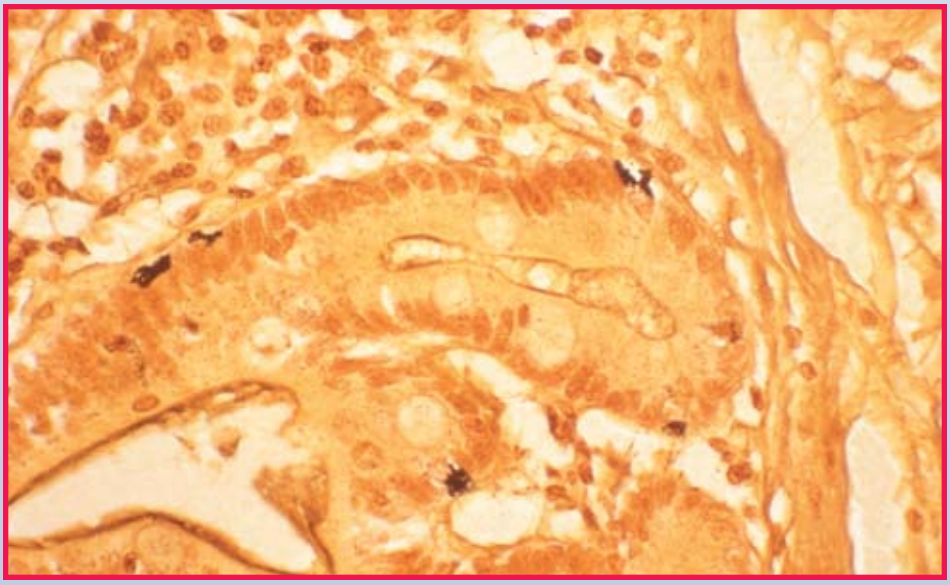
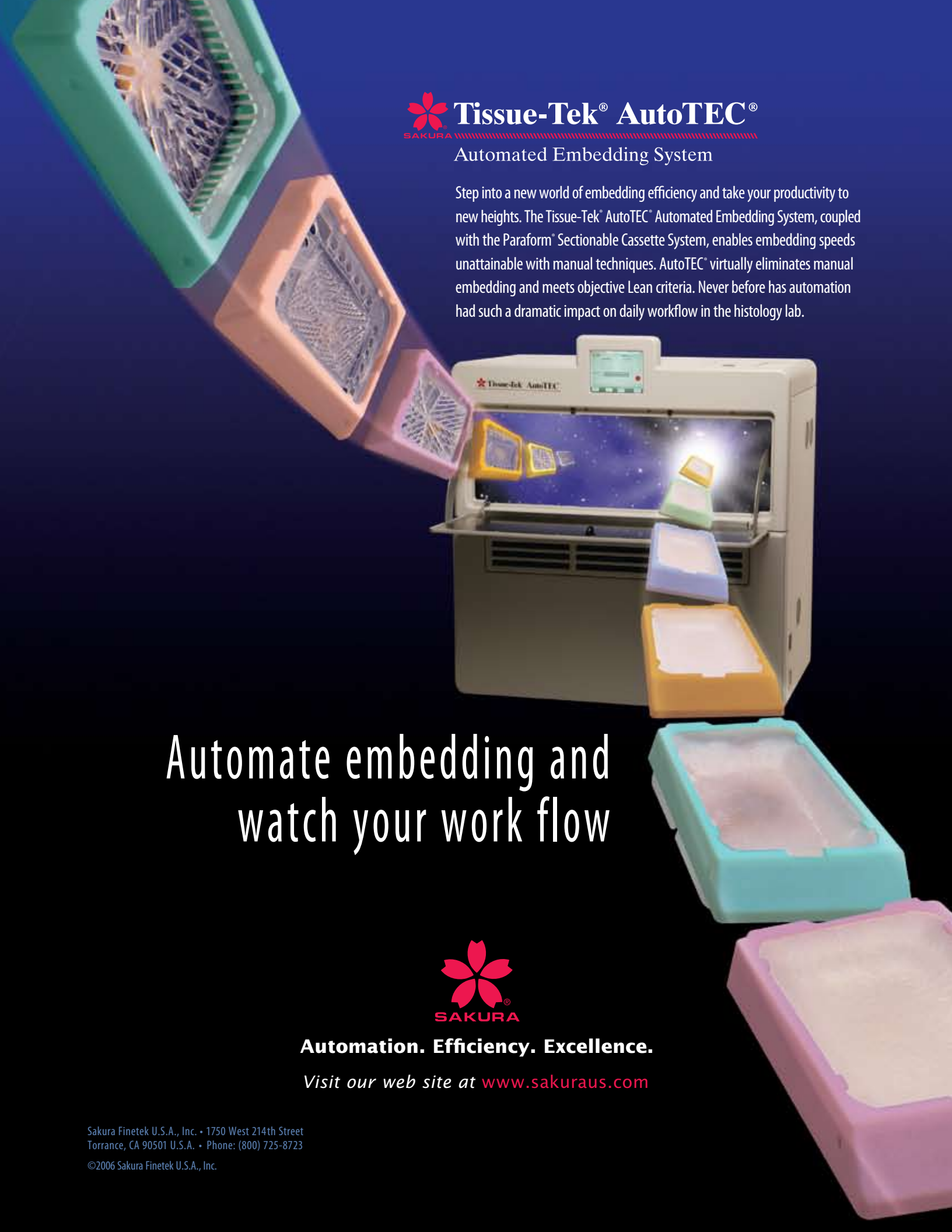


Fig. 3. A section of small bowel stained using Churukian's method for argyrophilic cells. Argyrophilic cells are stained black. 200X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Potassium dichromate is an artefact pigment that appears as a fine yellow deposit. It may be removed by treatment with acid alcohol.

Starch or *talcum powder* is a pigment usually introduced by talc from the gloves of a surgeon, nurse, or pathologist. This pigmentation is PAS- and GMS-positive and can be easily identified by its characteristic appearance, which includes a "Maltese cross" configuration when polarized.

Hematogenous Pigments

Hemoglobin is the only hematogenous pigment that is present in normal tissue, ie, the red blood cell. This is the oxygen-carrying conjugated protein of the red blood cell and consists of a colorless protein component and a pigmented portion containing iron. It occurs pathologically in renal casts of hemoglobinuria as droplets or granules of a yellow or yellow-brown color. It can be demonstrated by Lison's patent blue V method[1] (Fig. 1).

Hemosiderin is a breakdown product of hemoglobin and is thought to be composed of ferric iron and protein. It occurs in pathological conditions such as infarcts and in areas of hemorrhage and thrombosis, usually appearing within macrophages as yellow-brown granules. It can be easily demonstrated by Pearl's method[2] for ferric iron. This method is sometimes referred to as the Prussian blue reaction. The principle of this reaction (illustrated below) is that potassium iron ferrocyanide will form Prussian blue with reactive ferric salts in an acid solution. Dilute hydrochloric acid is sufficient to liberate loosely bound ferric iron, such as hemosiderin, from protein (Fig. 2).

Prussian Blue Reaction

$$K_4Fe(CN)_6 \cdot 3H_2O \xrightarrow{HCl} Fe_2[Fe(CN)_6]_3 + 12K$$

Potassium Ferrocyanide — Prussian Blue

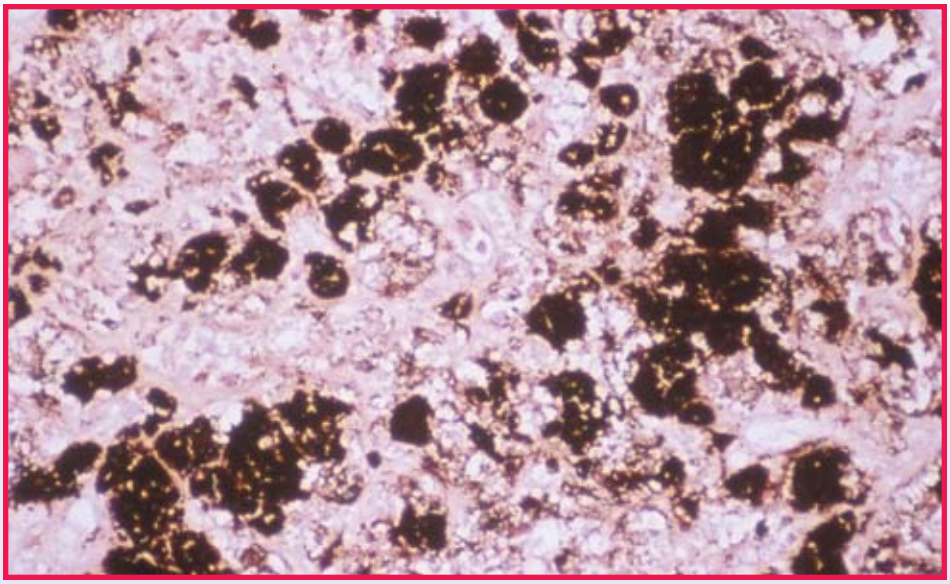

Fig. 4. A section of liver from a patient with malignant melanoma stained by the Fontana-Masson method for melanin. Melanin is stained black. 400X

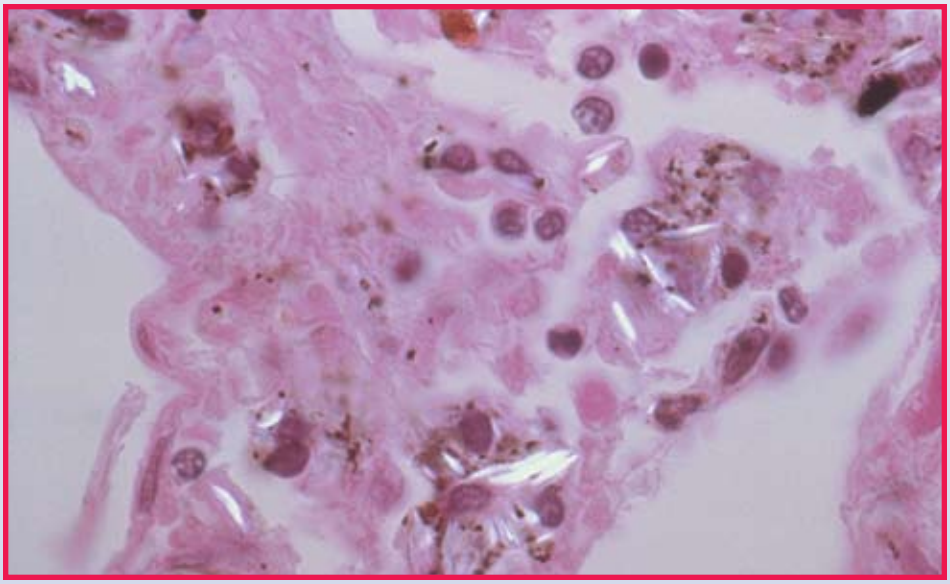

Fig. 5. A section of lung from a patient with silicosis stained with H&E as seen with polarized microscopy. Silica polarizes yellow-white. 400X

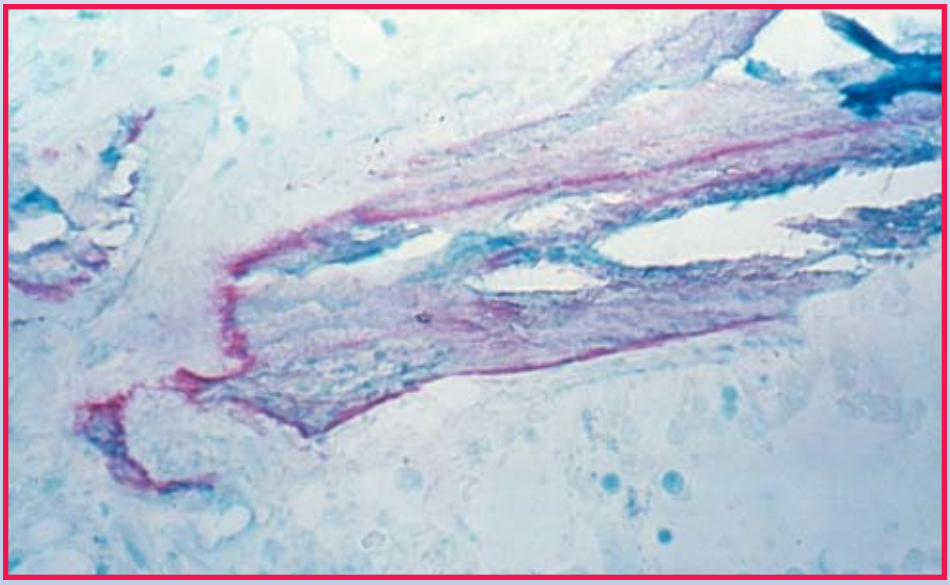

Fig. 6. An undecalcified glycol-embedded section of bone stained with Lillie's aluminon method for aluminum. Aluminum stains red. 200X

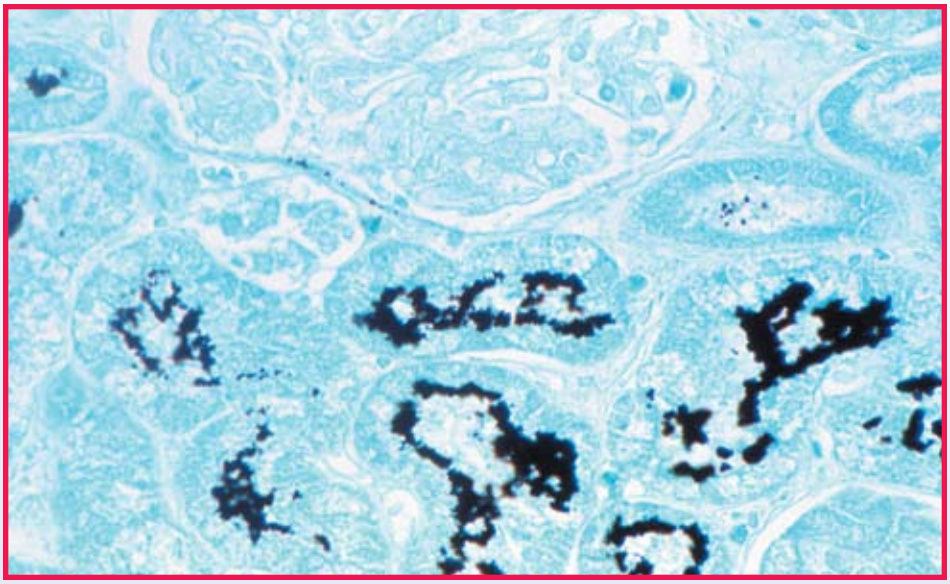

Fig. 7. A section of kidney from a patient with kidney stones stained with the von Kossa silver nitrate method. 100X

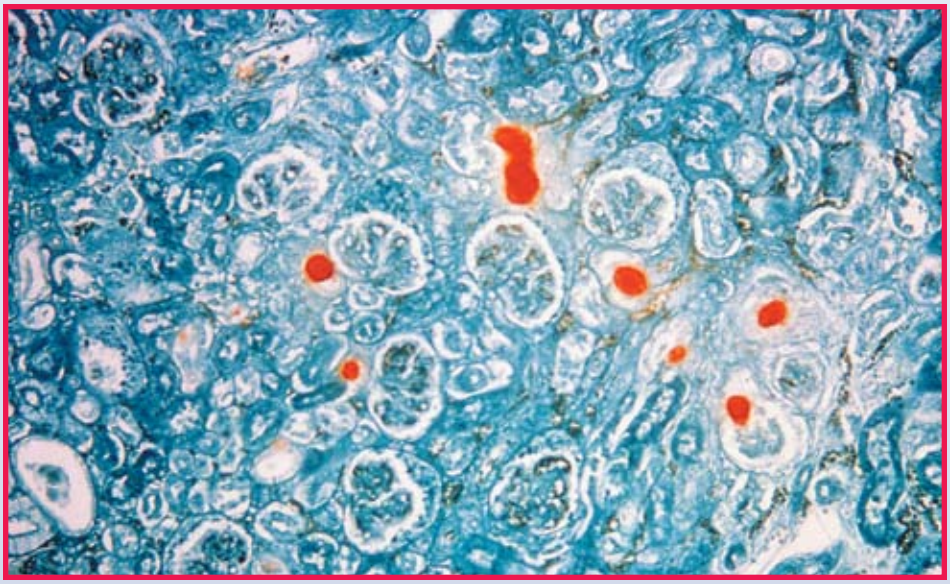

Fig. 8. A section of kidney from a patient with kidney stones stained with Dahl's alizarin red S method for calcium. Calcium stains red. 100X

Fig. 9. A section of kidney from a patient with kidney stones composed of calcium oxylate as seen with polarized microscopy. 400X

Hematoidin (bile pigment) is derived from red blood cells and occurs as yellowish or greenish granules or masses. It is chemically the same as bilirubin and can be demonstrated by Hall's method.[3]

Hemozoin (malaria pigment) is found in the malarial parasites and may be seen in the liver, spleen, bone marrow, lymph nodes, and brain capillaries. It is similar to formalin pigment, but may be differentiated from formalin pigment by its location and distribution. To remove this pigment, treat the section with 10% ammonium hydroxide in 70% alcohol for 5 to 15 minutes.

Autogenous Pigments

Argentaffin cells (enterochromaffin cells) are also known as Kulchitsky cells. They are found normally in the mucosa of the stomach, small and large bowel, and the appendix. They usually contain serotonin, which is a biogenic amine.

In addition to argentaffin cells, there are also argyrophil cells in the gastrointestinal mucosa. Argyrophil cells are also found in the pancreas, trachea, bronchi, and prostate. These cells, as well as enterochromaffin cells, are referred to as the APUD (amine precursor uptake and decarboxylation) system. The APUD cells contain some type of polypeptide hormone and/or biogenic amine.

Tumors derived from argentaffin or argyrophil cells are called apudomas or carcinoid tumors. Argentaffin cell tumors may give a positive reaction with the Fontana-Masson silver method and Schmorl reaction. Argyrophil cell tumors may be demonstrated by the Grimelius and Pascual silver stain methods. For best results, Churukian's microwave modification of Pascual's method[4] is recommended (Fig. 3). However, the monoclonal antibodies chromogranin, synaptophysin, and PGP 9.5 have, for the most part, replaced the histochemical methods for demonstrating apudomas.

Argentaffin means having the ability to reduce silver salts without light or the aid of any reducing agent. Argy-

rophil, on the other hand, indicates that the tissue element can be impregnated with silver, but light or a reducing agent is required to produce the black deposit of metallic silver.

Melanin, another autogenous pigment, occurs normally as yellow-brown to black granules within melanocytes in hair, skin, and the eye. It may be found throughout the body in most, but not all (amelanotic melanomas) malignant melanomas. This pigment may be demonstrated by the Fontana-Masson (Fig. 4) and Churukian silver stain methods[5,6] and by the Schmorl reaction.[7,8] However, because other pigments give a positive reaction with these methods, a melanin bleach procedure with potassium permanganate and oxalic acid should also be done. The monoclonal antibodies HMB-45, Melan A, and NSE have good specificity for demonstrating melanin in most melanomas.

Chromaffin is an autogenous pigment normally found in the cells of the adrenal medulla that appears as dark-brown granular material. It may occur in tumors of the adrenal medulla (pheochromocytomas). Fixation in formalin is not recommended, and fixatives containing alcohol, mercury bichloride, or acetic acid should be avoided. Orth's solution or other dichromate-containing fixatives are recommended. Chromaffin may be demonstrated by Schmorl's reaction[7,8] and it is PAS-positive.

The last of the autogenous pigments is *lipofuscin,* which is known as "wear and tear pigment" or "brown atrophy." It occurs as a fine yellow-brown granular pigment in the liver, heart muscle, adrenals, and ganglion cells. It appears to be a heterogenous group, and Pearse traces it from a lipid precursor, which on progressive oxidation, gives varying reactions. It is often positive with stains for lipids, the performic acid-Schiff reaction, Schmorl's reaction, the Fontana-Masson silver stain method,[5] and occasionally with the PAS method.

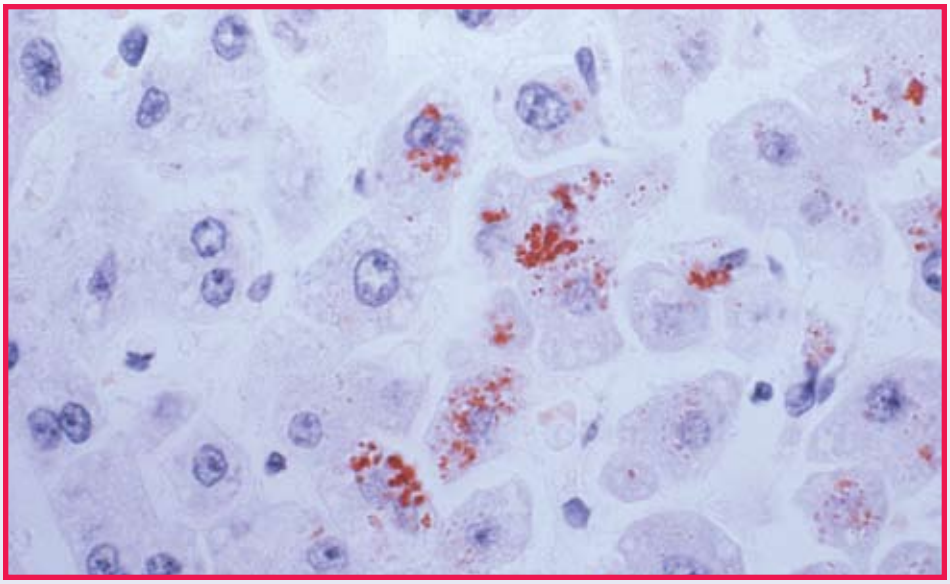

Fig. 10. A section of fetal liver in the third trimester stained with Lindquist's rhodanine method for copper. Copper is stained red. 400X

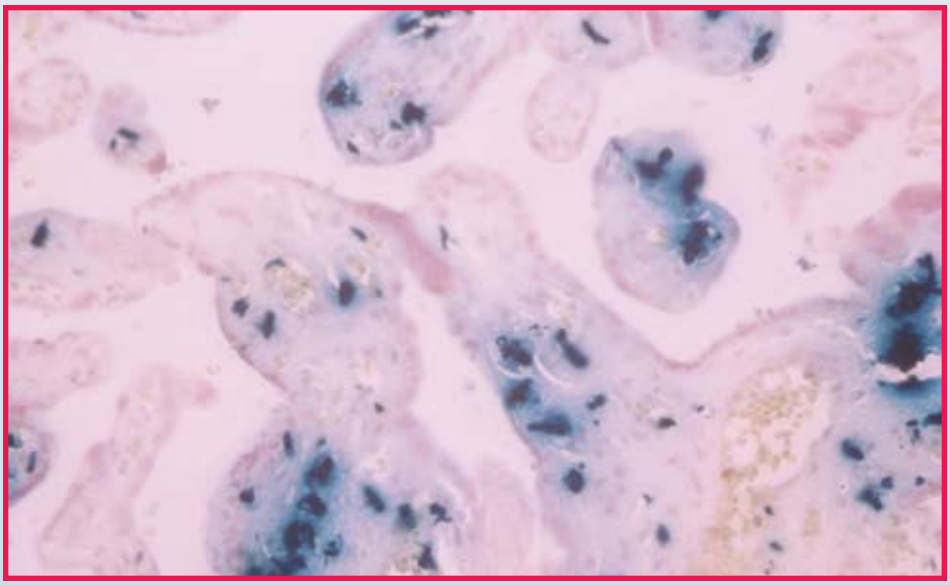

Fig. 11. A section of placenta stained with Lillie's method for ferrous iron that is known as Turnbull's blue. 200X

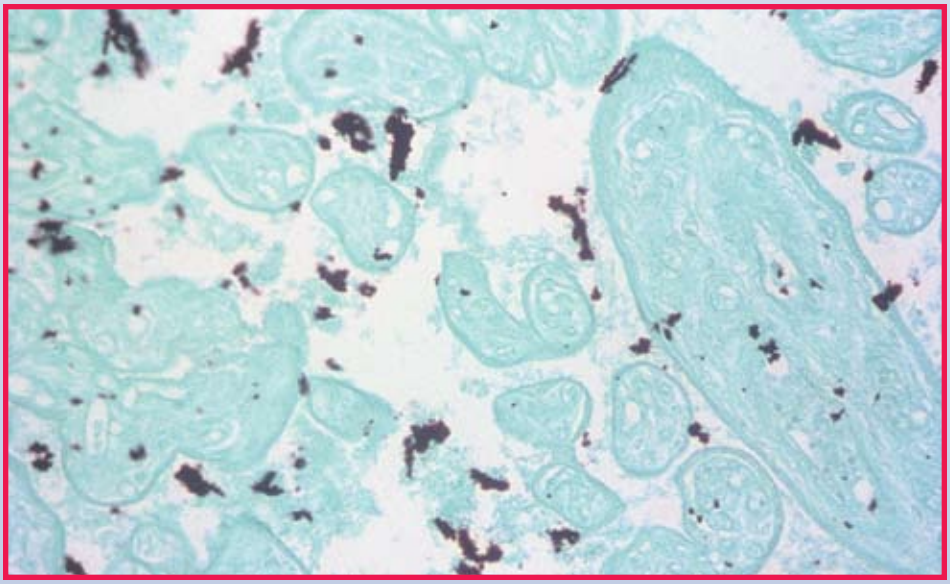

Fig. 12. A section of placenta stained with Lillie's rhodizonate method for lead. Lead is stained black. 200X

Exogenous Pigments

Carbon occurs as jet-black granules or masses and is sometimes referred to as anthracotic pigment. It is usually seen in the lungs and associated lymph nodes. Carbon is distinguished from other pigments by its insolubility in concentrated sulfuric acid.

Silica is found most commonly in the lungs and associated lymph nodes of stone grinders, where it manifests as silicosis. In coal miners, it occurs together with carbon, which presents as anthracosis. It occurs as grayish crystals that are birefringent. Silica pigment may be demonstrated by its resistance to microincineration, and by the fact that it is birefringent (Fig. 5).

Asbestos is a special form of silica and is found in the lungs and associated lymph nodes of asbestos workers. It has a characteristic appearance that somewhat resembles a barbell shape and gives a positive reaction with the methods for ferric iron. It is not anisotropic and, therefore, cannot be demonstrated by polarized light. The characteristic shape and light brown color make it easy to identify in routine hematoxylin and eosin sections.

Silver may be found in skin, kidneys, or other parts of the body as a result of medical treatment. Its presence among silver nitrate workers presents an occupational hazard, sometimes resulting in a condition known as argyria. It occurs as a brown or black granular deposit and may be removed from the tissue specimen by treatment with Lugol's iodine and hypo.

Tattoo pigment encompasses a great variety of colored pigments. They are usually confined to skin that has been tattooed, but may be found in associated lymph nodes.

Metals

Aluminum is a metal not normally found in tissues. It has been reported in patients who have had Alzheimer's disease and has been detected in bones of patients who have undergone prolonged renal dialysis. Aluminum may be identified with the Lillie's aluminon (aurine tricarboxylic acid) method[9] (Fig. 6).

Calcium salts are normally present in bone and teeth but, in some pathologic conditions, deposits of calcium and areas of calcification are formed in tissues normally devoid of these salts. The calcium salts normally found in animal tissues are calcium carbonate, calcium oxalate, and calcium phosphate. Calcium will stain blue with alum hematoxylins but more specific methods should be employed for their definite identification. Calcium can be identified with von Kossa's silver nitrate[10] (Fig. 7) and Dahl's alizarin red S[11] (Fig. 8) methods. However, alizarin red S will not stain calcium oxalate, which is demonstrated by the von Kossa method. Because calcium oxalate is birefringent, it can be identified easily by polarization (Fig. 9).

Copper in small amounts is present in the liver and is hardly detectable by standard staining methods, except in third trimester fetal liver. Excessive amounts of copper may occur in various pathologic conditions such as Wilson's disease, primary biliary cirrhosis, and other diseases of the liver. Copper can be detected by the rubeanic acid and rhodanine methods; Lindquist rhodanine method is the method of choice because of its greater sensitivity and specificity[12,13] (Fig. 10).

Ferric iron is a metal that has already been discussed in the Hematogenous Pigments section.

Ferrous iron is a metal that is seldom seen in tissues. It can be demonstrated with Lillie's method[14] for ferrous iron that substitutes potassium ferricyanide in place of potassium ferrocyanide in the staining solution (Fig. 11).

Lead occurs in chronic lead poisoning where it can be deposited in various tissues, particularly bone and kidney tables. It can be demonstrated by Lillie's rhodizonate method[15] for lead salts as described by Bancroft and Gamble[16] (Fig. 12).

Conclusion

The actual staining methods for several of the pigments and minerals to which this article refers can be found in the chapter titled "Pigments and Minerals" in *Theory and Practice of Histological Techniques,* 5th edition, 2002, edited by John D. Bancroft and Marilyn Gamble, published by Churchill-Livingstone. The 6th edition of this book will be published in 2007 and will contain an updated and revised chapter on pigments and minerals. Staining methods for pigments and minerals can also be found in *Manual of the Special Stains Laboratory,* Department of Pathology, University of Rochester Medical Center, 10th edition, 2005, edited by Charles J. Churukian.

Acknowledgment

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Fixatives: Coagulative vs Non-Coagulative or Is It Additive vs Non-Additive?

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One area of the ASCP Board of Registry HT and HTL exams that always causes confusion is how to classify fixatives. Some of the categories are easy—Is it an aldehyde or a metal salt fixative? Is it better for nuclear or cytoplasmic preservation? Is it physical or chemical?

But then there are the categories of coagulative/non-coagulative and additive/non-additive. There is a lot of confusion surrounding these terms. Some of it stems from the fact that various textbooks classify the fixatives differently. Some of the confusion falls into the category of what I would call "fading out of an old concept, but the new one hasn't caught on yet."

Coagulative vs Non-coagulative

This theory goes back for decades. In 1958, JR Baker explained in the *Principles of Biological Microtechniques* that coagulative fixatives create a mesh network of the proteins, thus allowing other solutions to easily penetrate through the mesh. Non-coagulative fixatives, according to Baker, cause the proteins to form a gel, thus making it harder for solutions to penetrate. Nowadays, I think that most people consider these two concepts to be an observation of what the tissue proteins seem to be doing physically, rather than what is actually happening chemically.

An experiment I do with my students is very easily reproduced in any histology laboratory. Get about 6 or 8 jars with lids (clear if possible, as it's easier to watch the reactions). Put 20 mL of a different fixative in each jar—whatever you have: 10% formalin, glutaraldehyde, 100% alcohol, 70% alcohol, acetone, acetic acid, Bouin's, zinc formalin, Hollandes, alcoholic formalin, proprietary fixatives. Take a raw egg at room temperature and separate out the yolk, saving the whites in a beaker. Egg whites (albumin) are made up of all protein. Using a pipette, place 2 mL of egg white in each jar with the different fixatives. Then see what happens. Watch the change in consistency of the egg white in the different fixatives and note the time frame during which any changes occur (Figs. 1, 2, 3).

Fig. 1. The egg white hardens up and turns white almost immediately in the 100% alcohol and acetone, similar to raw egg on a hot skillet. The photo was taken 10 minutes after the egg white was placed in these two solutions, but the change was seen within 1 minute. Within a couple of hours, the egg white was so hard it was brittle and would break apart when touched with a wooden stick.

Fig. 2. In the zinc formalin and the Bouin's, the egg white hardens a little slower than it does in the pure ethanol or acetone, but the egg white has a consistency of a soft boiled egg after 10 minutes of fixation. There is less hardening in these fixatives than in the pure ethanol or acetone. Hollandes, mercuric fixatives, and 100% acetic acid behave similarly.

Fig. 3. The egg white is at the bottom end of the wooden sticks but cannot be seen. The egg whites have not changed color or hardened in the first 10 minutes. The egg white in the 10% formalin looks and acts just like we had put the egg white into room temperature water. It remains the same consistency as raw egg white. Even by the next morning, the egg white wasn't visible or any harder. It never "dissolved" into the formalin, but it remained clear and swirlable. Egg white in glutaraldehyde behaved similarly. In the alcoholic formalin, the egg white remained the same for the first few hours, but eventually the 70% alcohol caused the egg to slightly turn white in some areas and harden slightly, similar to the changes first seen when egg white is first placed in simmering water to poach. In the first minute of poaching, the egg white takes on a slight white color in some areas, and it becomes a little harder in some areas, but it would be very difficult to take the egg out of the water with a slotted spoon in that first minute. Alcoholic formalin-fixed egg white is similar to this state; however, the alcoholic formalin never hardens the egg completely, no matter how long it is in this fixative.

Proprietary fixatives give a variety of different reactions, depending upon their chemical composition and their percentages. Therefore, based on experiments like this, fixative chemicals can be classified as:

Non-coagulative:

- aldehydes (formaldehyde/formalin, glutaraldehyde, paraformaldehyde)
- potassium dichromate
- osmium tetroxide/osmic acid (fixes lipids, not proteins)

Coagulative:

- all other fixative chemicals
  - metallic salts (mercuric oxide, zinc sulfate or chloride, cupric sulfate)
  - alcohols (ethyl, methyl, etc.)
  - acetone
  - picric acid

Acetic acid (and other dilute acids) were considered coagulative for nucleic acids, but non-coagulative for cytoplasmic proteins. This chemical is categorized differently in various textbooks. I usually classify it as coagulative, because the egg white did harden in our experiment.

However, many of the fixatives used are mixtures of chemicals. If there is one chemical that is coagulative, then the mixture is considered coagulative, even if there are 1, 2, or 3 other chemicals that are non-coagulative. So, for a fixative mixture to be considered non-coagulative, *all* the chemicals must be from the above non-coagulative list. Orth solution contains potassium dichromate and formaldehyde, both of which are non-coagulative, so Orth solution is a non-coagulative mixture. This can be verified by putting the egg white in the Orth solution; the egg white does not harden. Helly solution, on the other hand, contains potassium dichromate, formaldehyde, and mercuric chloride. Even though both potassium dichromate and formaldehyde are non-coagulative (just like in the Orth solution), the Helly mixture is considered coagulative because it contains mercuric chloride, which is an agent that coagulates protein. And in fact, the egg white will harden in the Helly fixative mixture.

Please note: water and buffering salts are *NOT* considered to be either coagulative or non-coagulative. When you look at fixative mixture formulas, do not consider these.

Additive vs Non-additive

Part of the problem with the terms coagulative and non-coagulative is that we know that even though the egg white does *not* look like it is hardening in a non-coagulative fixative such as formalin, we *do* know there is chemical bonding going on between the egg albumin proteins and the fixative. It is a fact that different chemical fixatives bind differently and stains react differently. This newer concept of additive and non-additive seems to be very relevant for histotechs.

Additive fixatives become physically bound (cross-linked) to something in the tissue, such as proteins, lipids, or nucleic acids, and are usually very difficult to remove from the tissue. Formaldehyde is a negatively charged chemical. It will cross-link with the positively charged amino acids in the tissue. There are a lot of positively charged amino acids in the cytoplasm and very few in the nucleus, which is why formalin is not a particularly good nuclear fixative. The dichromate part of potassium dichromate is also negatively charged, making it better at cross-linking and preserving cytoplasm than nuclei. On the other hand, most of the other metal salts used in fixation are positively charged

(zinc chloride and sulfate, mercuric chloride, cupric acetate). These metal salts will bind (cross-link) with the negative amino acids in the tissue. There are more negative charges in the nuclei than in the cytoplasm, which is why these metal salts fix the nuclei much better than formaldehyde. This also helps to explain why fixation in different chemicals will contribute to different immunohistochemical (IHC) staining results, or may require different epitope recovery methods, because different amino acids and proteins are being cross-linked.

There is also a different shift in charges in the tissue, depending upon which fixative chemical is used. In Fig. 4, the protein strands (green) are made up of 6 positively charged amino acids and 6 negatively charged amino acids. As it appears, the tissue has zero net charge. In Fig. 4A, formaldehyde (yellow circle), which has a negative charge, will bind with the positively charged amino acids. This leaves 4 positively charged and 6 negatively charged amino acids, so the tissue has a net negative charge, and will bind more strongly with positively charged dyes. In Fig. 4B, the positively charged metallic salts (red square) such as zinc, mercury, and copper, will bind with the negatively charged amino acids, leaving the tissues with 6 positively charged and 4 negatively charged amino acids. The tissue is now more positively charged and will bind more strongly with negatively charged dyes. This helps to explain why, when you have two tissues from the same patient, one fixed in zinc formalin solution and the other fixed in plain 10% formalin, they will display different staining characteristics in an H&E stain. The zinc formalin is much more pink. The zinc-fixed tissue is more positively charged, which enables it to bind a greater number of the negatively charged eosin dye molecules. In comparison, the formalin-fixed tissue is now more negatively charged and has fewer binding sites for the negatively charged eosin dye molecule, so it will appear a paler pink than the zinc-fixed tissue.

Rather than provide specific examples of the additive fixative chemicals at this point, additive fixatives will be defined as any fixative that is *NOT* a non-additive fixative. We'll talk more about this at the end of this article.

Non-additive fixatives fall into two categories. One type of non-additive fixative is

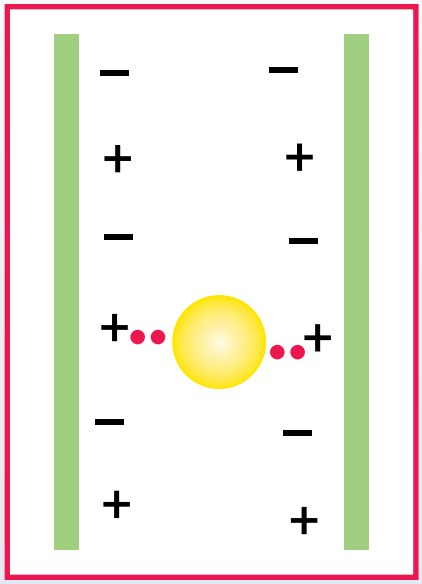

Fig. 4A. Formaldehyde

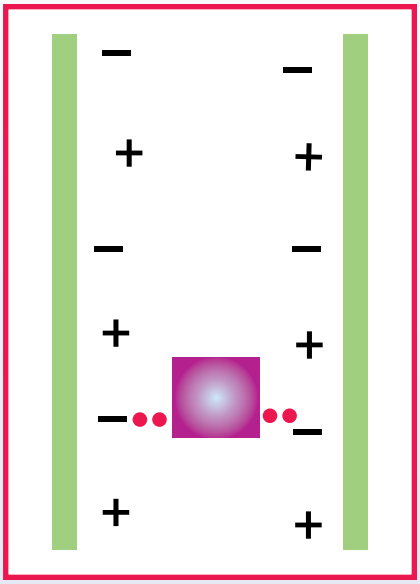

Fig. 4B. Metallic salt

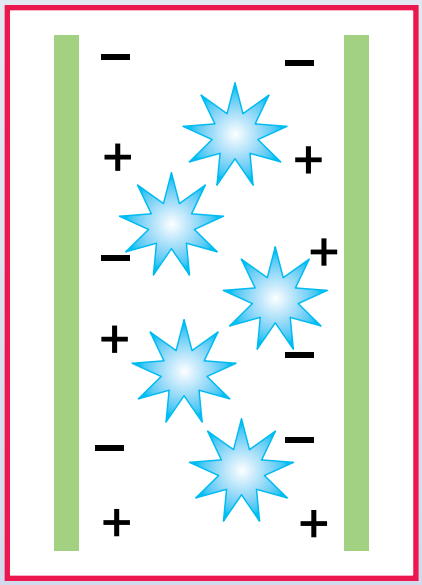

Fig. 5A. Amino acids with water

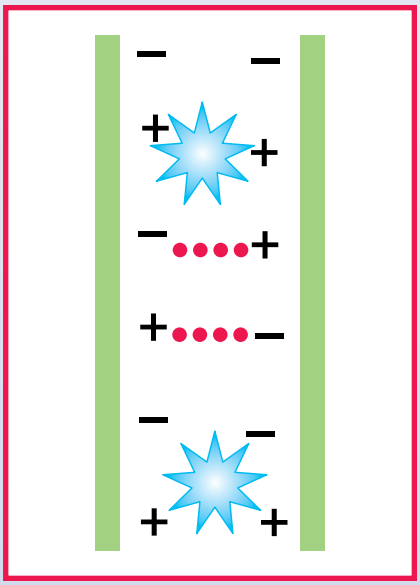

Fig. 5B. Amino acids cross-link; protein shrinks

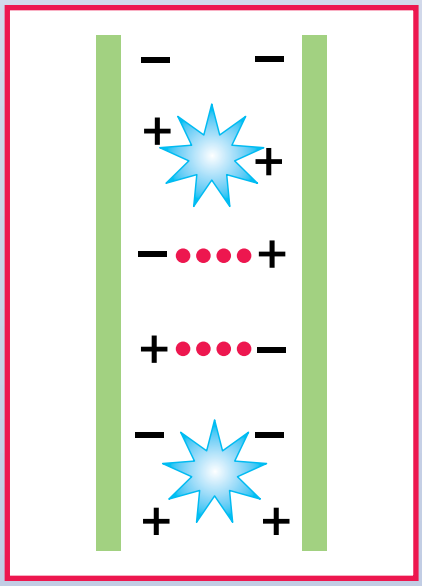

Fig. 6A. Normal protein cross-links

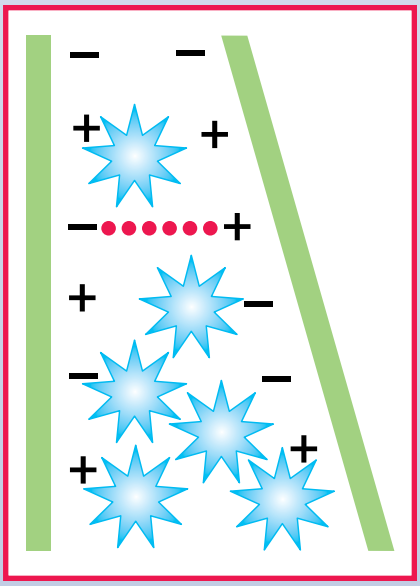

Fig. 6B. Acid effect breaks cross-links—water added; protein swells

what I call the *dehydrants,* such as alcohols (methanol, ethanol, isopropanol, etc.), acetone, and others. These are the chemicals that remove water from tissue—from between the protein layers. These bound and unbound water molecules help to keep the proteins separated from each other (Fig. 5A). When these protein layers are separated, very few of the positively and negatively charged amino acids are near each other, so very few will cross-link on their own. When the dehydrants remove the water, the protein layers move closer together, which is why we see shrinkage of the tissues, and the tissues are denser to section. Also, now that the protein layers are closer together, the charged amino acids are closer together. These formerly separated amino acids can cross-link—positive to negative (Fig. 5B). If you notice, the dehydrants have *not* chemically attached the tissue. The dehydrants have simply removed the water, allowing the proteins to shrink and come closer together, thus allowing the positively and negatively charged amino acids to electrostatically bind to each other.

As you can see in Fig. 5B, if we start with 6 positively charged amino acids and 6 negatively charged amino acids (net zero charge), with the two cross-linking amino acids, we now have 4 positively charged and 4 negatively charged amino acids, so the net charge is still zero.

Now compare H&E staining of two formalin-fixed tissues—one well fixed, the other underfixed in formalin (or fixed in alcoholic formalin). In the tissue fixed with formalin, the formaldehyde cross-links help to stabilize the tissue proteins. Even when the tissue is subsequently placed in a dehydrating solution on the tissue processor and water is removed, the tissue will not shrink much because the formaldehyde cross-links are stabilizing the proteins and keeping them apart. If there are not enough formaldehyde cross-links, such as in underfixed tissue or tissue placed in alcoholic formalin first, where alcohol penetrates faster than the formalin, then the tissue is not stabilized, the proteins shrink due to the alcohol removing the water, the positive and negative amino acids are cross-linking with each other, and *then* the formaldehyde comes in and cross-links with whichever negatively charged amino acids are left, in the shrunken configuration caused by the alcohols. Therefore, these tissues are more alcohol fixed than formaldehyde fixed. As a result, there will be more distortion, the histology stains will have different charges to link to, and the IHC will react differently from tissue that was well fixed in formalin before being placed into a dehydrant.

The second type of non-additive fixative is the *acids,* with acetic acid as the primary acid used in fixative mixtures. Acids have the opposite effect of the dehydrants. As you can see in Fig. 6A, in tissue that is unfixed, even with all the bound and unbound water, there are some amino acids that can be close to each other and can loosely cross-link. Between these amino acid cross-links are other amino acids that are charged but are "hidden" from the water. Acids break apart these amino acid cross-links. Now these formerly cross-linked amino acids, as well as the hidden amino acids, are available to bind with water (Fig. 6B). This causes the proteins to swell. But again, these acids are *not* chemically bound to the tissue.

Some of the chemicals can cause shrinkage, so an acid is usually added in the fixative mixture to counteract the shrinkage (acetic acid will cause cells to swell). Because some chemicals only fix cytoplasm, a fixative mixture might add a chemical that fixes just the nuclei.

So how do we classify mixtures as being additive or non-additive? Well, we don't. We cannot say a mixture is either additive or non-additive. We have to say what each chemical is doing. In Helly, the mercuric chloride is additive, the potassium dichromate is additive, and formaldehyde is additive. In Zenker, which is similar to Helly, the mercuric chloride is additive, the potassium dichromate is additive, and the acetic acid is non-additive.

Again, just like with coagulative and non-coagulative, the water and buffering salts are not included in the additive and non-additive determination.

Acknowledgment

I would like to acknowledge Jerry Fredenburgh, Richard Dapson, Ada Feldman, and Dorothy Cummings for all I was able to learn from their invaluable fixation workshops.

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Helpful Hints to Remember Which Is Which

It's easier to learn the exceptions under the "non" categories. The rest of the chemicals are classified as "everything else."

Non-coagulative vs Coagulative		
Chemical Type	**Code Word**	**Examples**
Non-coagulative	APDOT	Aldehydes (all of them), potassium dichromate, osmium tetroxide
Coagulative	--	All the rest of the chemical fixatives
Mixture	--	If there is even one coagulative chemical, then the mixture is coagulative. For the mixture to be considered non-coagulative, all chemicals in the mixture must be non-coagulative.

Non-additive vs Additive		
Chemical Type	**Code Word**	**Examples**
Non-additive	3 As	Alcohols (all types), acetone, acids (all types)
Additive	--	All the rest of the chemical fixatives
Mixture	--	Cannot categorize mixtures as either additive or non-additive; must categorize each chemical separately

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Trichrome — Three Colors, Six Ways

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Abstract
The Masson's trichrome stain is often the most tedious routine special stain performed in our lab. We perform Masson's trichrome staining for the evaluation of fibrosis in various disease processes. It is a time-consuming technique due to long incubations and the myriad of solutions that must be prepared. Also, the color contrast between red and blue may not be ideal for distinguishing different tissue structures. In addition, the requirement that Bouin's solution be used as a postfixation mordant to achieve acceptable staining results with the Masson's trichrome stain creates safety concerns due to its picric acid content.

For these reasons, we explored several modifications including:

- The use of Bouin's alternatives including Bouin's 2000™, citrate buffer, and Gram's iodine to address safety concerns
- The use of Gomori's one-step trichrome to explore a less time-consuming method
- Substitution of fast green for aniline blue in the Masson's trichrome to explore whether this improves the aesthetic value of the stain

In this experiment we set out to demonstrate that the different trichrome methods have varying effects on staining results and interpretation, suggesting that incorrect implementation can lead to suboptimal results. Several of the methods tested yielded acceptable results, so method selection becomes a matter of cost, safety, and personal preference.

Materials and Methods
Pig hearts were fixed in 10% neutral buffered formalin (NBF), sectioned at 5 µm, and stained using the following methods.

Bouin's 2000: Bouin's 2000 (American Master Tech Scientific Inc., Lodi, CA) was preheated in a 60°C oven. Slides were deparaffinized as outlined in Table 1, and then placed in Bouin's 2000 in the 60°C oven for 1 hour. Slides were removed from Bouin's 2000 and rinsed for 5 minutes. Slides were then placed on the Sakura DRS™ Stainer (Sakura Finetek U.S.A., Inc., Torrance, CA) using the protocol outlined in Table 2.

Table 1 – Hydration Protocol

Deparaffinize	
Xylene	5 minutes
Xylene	5 minutes
100% alcohol	2 minutes
100% alcohol	2 minutes
95% alcohol	2 minutes
95% alcohol	2 minutes
Tap water rinse	1 minute

Gram's Iodine: Gram's iodine was preheated in a 60°C oven. Slides were deparaffinized (Table 1) then placed in Gram's iodine in a 60°C oven for 1 hour. Slides were removed from Gram's iodine and rinsed for 5 minutes. Slides were then placed on the Sakura DRS™ Stainer using the protocol outlined in Table 2.

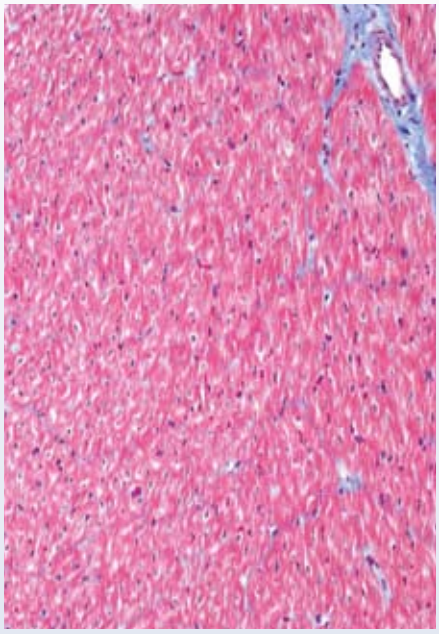
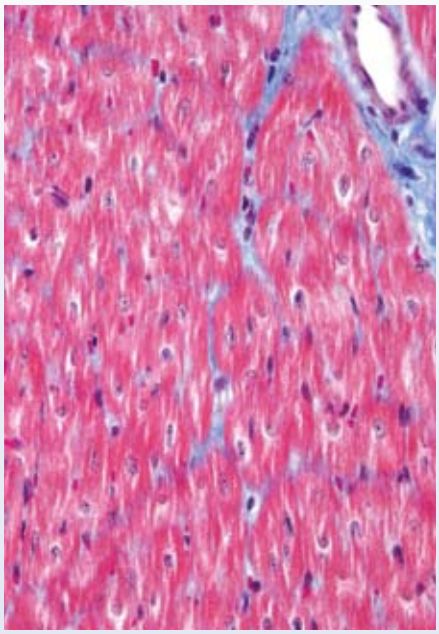
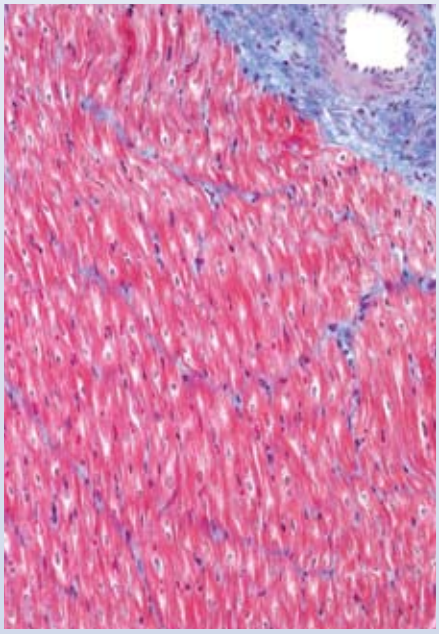
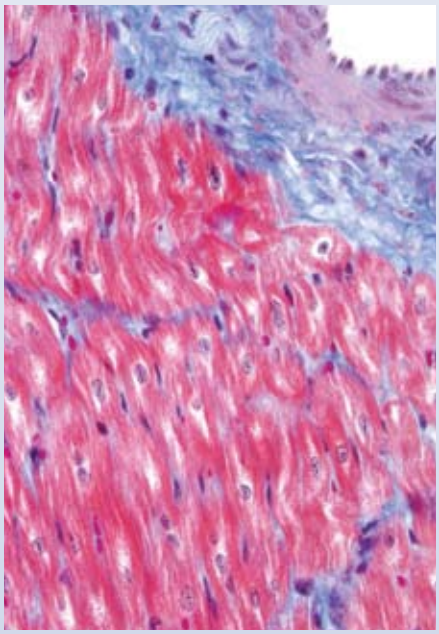
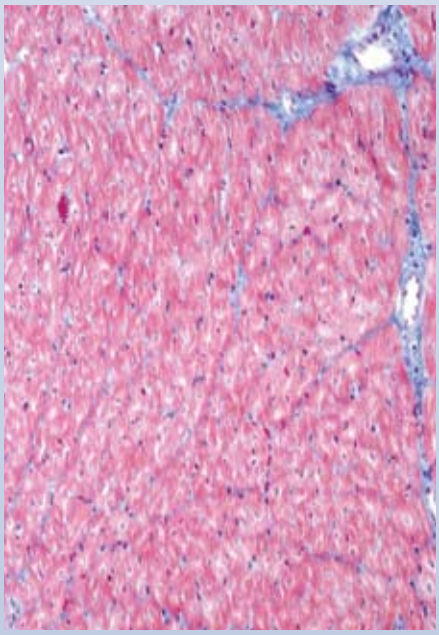
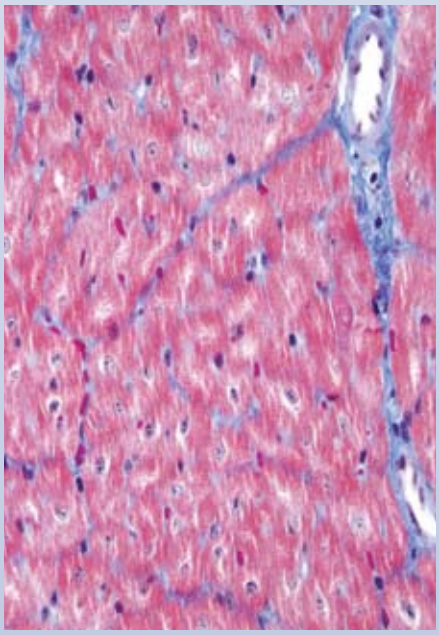
Citric Acid-Sodium Citrate Buffer: 0.01M citric acid-sodium citrate buffer pH 6.0 was prepared according to a protocol in Kiernan, outlined in Table 3. In order to obtain a pH of 6.0, 9.5 mL of solution A was added to 40.5 mL of solution B. In order to obtain a 0.01M solution, this ratio of A to B was diluted with 9 parts deionized water (50 mL A:B plus 450 mL H_2O). The pH was then checked using a Mettler Toledo SevenEasy™ pH meter (Mettler Toledo, Columbus, OH), and the pH was adjusted accordingly using either 10N sodium hydroxide to bring the pH up or 10N hydrochloric acid to bring the pH down.

0.01M citric acid-sodium citrate buffer was preheated in a 60°C oven. Slides were deparaffinized (Table 1), and placed in citric acid-sodium citrate buffer in a 60°C oven for 1 hour. Slides were removed from citric acid-sodium citrate buffer and rinsed for 1 hour. Slides were then placed on the Sakura DRS™ Stainer using the protocol outlined in Table 2.

Gomori's One-Step: Bouin's solution (Richard Allan Scientific, Kalamazoo, MI) was preheated in a 60°C oven. Slides were deparaffinized (Table 1) and placed in the heated Bouin's solution for 1 hour. Slides were removed and rinsed for 1 hour to remove all traces of picric acid. Slides were stained using

Table 2 – Masson's Trichrome Stain

Trichrome	
Weigert's iron hematoxylin	10 minutes
Tap water rinse	10 minutes
Biebrich scarlet/acid fuchsin	2 minutes
Tap water rinse	2 minutes
Phosphotungstic/phosphomolybdic acid	13 minutes
Aniline blue	5 minutes
Tap water rinse	3 minutes
1% Glacial acetic acid	4 minutes
95% reagent alcohol	1 minute
95% reagent alcohol	1 minute
100% reagent alcohol	1 minute
100% reagent alcohol	1 minute
Xylene	1 minute
Xylene	1 minute

	Pig Heart 20X	Pig Heart 40X	
Bouin's 2000			**Advantages** • No picric acid **Disadvantages** • Poor cellular morphology
Gram's Iodine			**Advantages** • No picric acid **Disadvantages** • Iodine releases fumes when heated • Noted tinctorial differences • Not crisp
Citrate Buffer			**Advantages** • No picric acid • Easy sink disposal **Disadvantages** • Pale staining: challenging for pathologist to read

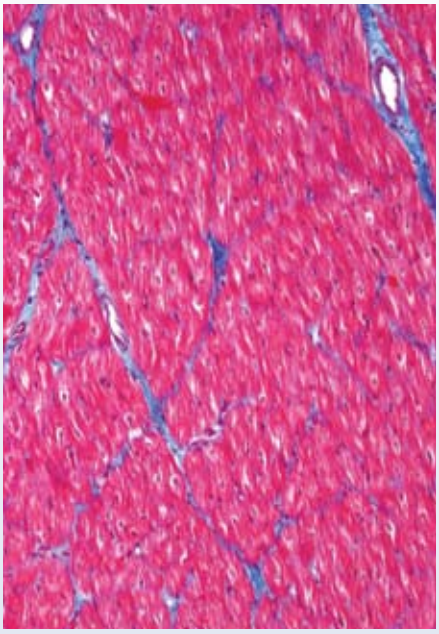
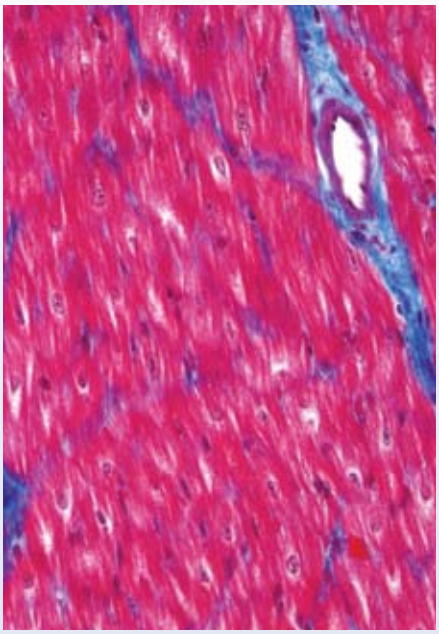
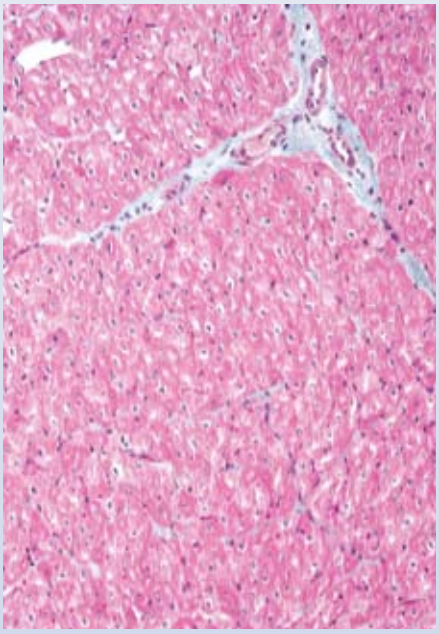
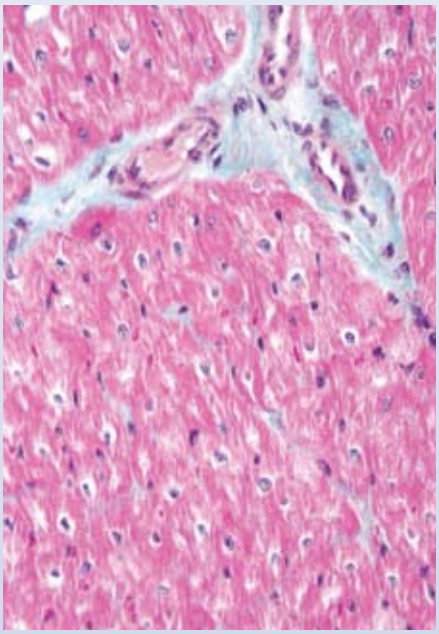
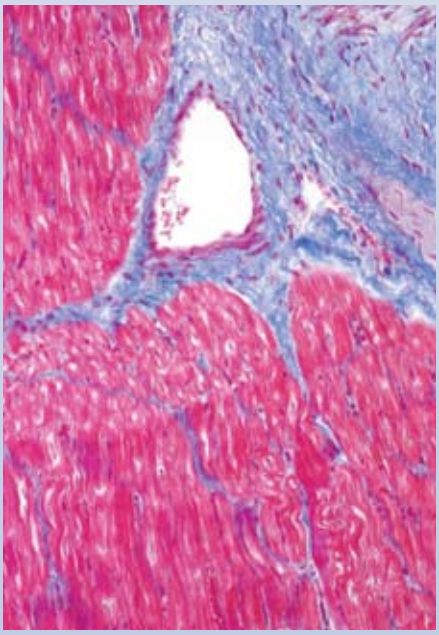
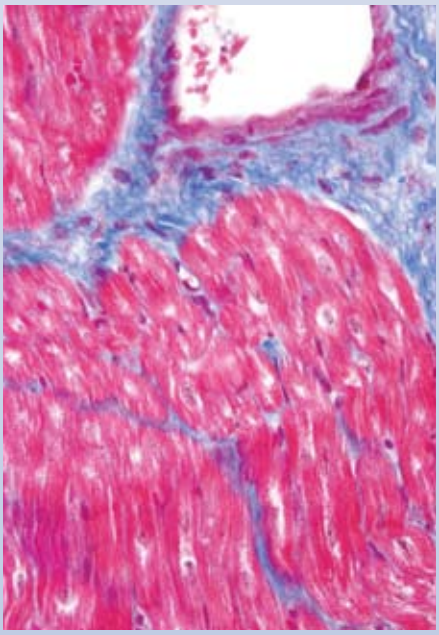
	Pig Heart 20X	Pig Heart 40X	
Gomori's One-Step			**Advantages** • More efficient protocol • Bright color • Good contrast between red and blue • Decreased waste **Disadvantages** • None noted
Fast Green			**Advantages** • None noted **Disadvantages** • Not suitable for diagnostic purposes due to poor contrast between red and green
Routine Masson's Trichrome			**Advantages** • Routinely run • Semi-automated • Pathologist approved **Disadvantages** • Use of Bouin's fluid as a postmordant • Lengthy protocol

Table 3 – Citric Acid-Sodium Citrate Buffer

Solution A: 0.1M Citric Acid	
Citric acid, anhydrous	19.2g
Reverse osmosis/distilled water	1000 mL
Solution B: 0.1M Sodium Citrate	
Sodium citrate, dihydrate	29.4 g
Reverse osmosis/distilled water	1000 mL

Gomori's one-step method (PolyScientific R&D Corp, Bayshore, NY) as shown in Table 4.

Routine Masson's Trichrome: Bouin's solution was preheated in a 60°C oven. Slides were deparaffinized (Table 1) and placed in the heated Bouin's solution for 1 hour after which slides were removed and rinsed for 1 hour. Slides were then placed on the Sakura DRS™ Stainer using the protocol outlined in Table 2.

Substitution of Fast Green: Slides were pretreated in Bouin's solution as in the routine Masson's trichrome, then placed on the Sakura DRS™ Stainer, using the protocol outlined in Table 2, with the substitution of 2.5% fast green (Sigma Chemical, St. Louis, MO) for aniline blue in Step 6.

Photographs were taken at 20X and 40X on a Nikon Eclipse E600E600 upright microscope equipped with a Nikon DXM1200C digital camera (Nikon, Melville, NY).

Results

In this experiment we set out to determine which trichrome staining method would yield the best results, while also being advantageous in one or more of the following areas: safety, length of procedure, and visual contrast. While no single technique is ideal for all tissue, through experimentation using paraffin heart sections, we have demonstrated that several methods can yield valuable results. The following is an overview of the results of each method. It is important to mention that the lack of proper fixation can have a marked effect on staining. The pig heart tissue used in these experiments was inadequately fixed, which is likely to have contributed to the uneven staining in some sections.

Table 4 – Gomori's One-Step Trichrome Stain

Weigert's iron hematoxylin	10 minutes
Wash in water (no time specified in protocol, opted for 10 min)	10 minutes
Gomori's one-step trichrome with aniline blue	15 minutes
Acetic acid 0.5% aqueous	2 minutes
95% reagent alcohol (reagent alcohol substituted for ethyl alcohol listed in original protocol)	2 minutes
95% reagent alcohol	2 minutes
100% reagent alcohol	2 minutes
100% reagent alcohol	2 minutes
Xylene	2 minutes
Xylene	2 minutes

Routine Masson's trichrome: This is the standard protocol that our pathologists are accustomed to evaluating. Bouin's solution is used to mordant sections prior to staining. This technique provides even staining, good contrast, and is valuable for diagnostic purposes.

Routine Masson's trichrome with iodine mordant: Substituting iodine for Bouin's solution provides patchy tinctorial variation in staining. It lacks crisp contrast, making it inferior to the routine Masson's trichrome. However, it still demonstrates fibrosis and is sufficient for diagnostic purposes.

Routine Masson's trichrome with Bouin's 2000 mordant: This protocol yields less distinct cellular morphology. Blue staining is not robust and we observed artificial separation of myofibrils. Although inferior to the routine Masson's trichrome, this stain is suitable for diagnostic purposes.

Routine Masson's trichrome with fast green in place of aniline blue: The green staining is not distinct and does not provide enough of a contrast with the red pigment. Fibrosis is not as distinct at lower magnification, therefore, this protocol is not recommended for diagnostic purposes.

Routine Masson's trichrome with citric acid: The staining is uneven throughout the section. Both red and blue pigments are pale, making the slide difficult to read. While this protocol is not ideal, it still works for diagnostic purposes.

Gomori's one-step trichrome: This method exhibits the brightest staining and the best contrast and is of comparable readability to the routine trichrome. The fibrosis is clear and evident. Our pathologist feels that this is a visually pleasing stain, and would prefer it to Masson's trichrome for diagnostic purposes.

Conclusion

The Masson's trichrome stain is often the most tedious routine special stain performed in our laboratory. As such, we set out to find a modification that would improve the staining process in terms of safety, length of procedure, and staining result. Based on our experiments, we have concluded that there are several methods that can yield acceptable results. We have also concluded that tissue and fixation play a significant role in staining results. In this study of pig heart tissue, we demonstrated that the Gomori's one-step trichrome is an effective alternative to Masson's trichrome. It proved to be a method that takes less time to perform and provides excellent staining contrast.

Bibliography

Bancroft J, Stevens *A. Theory and Practice of Histological Techniques.* 4th ed. New York, NY: Churchill-Livingstone; 1996.

Carson F. *Histotechnology: A Self-Instructional Text.* Chicago, Ill: ASCP Press; 1997.

Kiernan JA. *Histological and Histochemical Methods: Theory and Practice.* 3rd ed. Hodder Headline Group; 1999:421.

PolyScientific R&D Corp. Reference Guide. 2002:33.

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January 19
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Safety in the Histology Laboratory"**
Speaker: Clifford M. Chapman, MS, HTL(ASCP), QIHC Histopathology Supervisor
Children's Hospital
Boston, MA

January 24
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"Communicating Across Generations"**
Speaker: Elaine Torossian, MS
William Beaumont Hospital
Royal Oak, MI

February 16
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Ready Or Not, Here it Comes: Microwave Technology"**
Speaker: Donna Willis, HT/HTL(ASCP)
North American Application Manager
Milestone Medical
Grand Prairie, TX

February 28
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"Tissue Identification for the Histotech"**
Speaker: Thomas S. Haas, DO, FCAP
Mercy Health Systems
Janesville, WI

March 2-3
Indiana Society for Histotechnology
Site: Indianapolis, IN
Contact: LaDonna Elpers
(812) 963-8287
Email: president@ishhome.org

March 9-10
NSH Region I Meeting
Site: Ft. Devens, Devens Common Center
Devens, MA
Contact: Clifford Chapman
(617) 335-0561
Email: Clifford.chapman@childrens.harvard.edu

March 10
Arkansas Society of Histotechnology
Site: Little Rock, AR
Contact: Melissa Hill
Email: mkh@nwapath.com

March 16
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"What is 21st Century Orthopaedic Research"**
Speaker: Larry J. Suva, PhD
Director, Center for Orthopaedic Research
Departments of Orthopaedic Surgery and Physiology and Biophysics
UAMS College of Medicine
University of Arkansas for Medical Sciences
Little Rock, AR

March 16
ASCP Teleconference
12:00 noon Central Time (800) 267-2727
Title: **"A Holistic Approach to Antigen Retrieval and IHC Amplification"**
Speaker: David Tacha, HTL(ASCP)
Biocare Medical
San Ramon, CA

March 16–17
New Jersey Society for Histotechnology Symposium
Site: Clarion Hotel & Conference Center
Cherry Hill, NJ
Contact: Joe Tamasi
(609) 818-3288
Email: joseph.tamasi@bms.com

March 22–24
NSH Region III Meeting
Site: CIIT Centers for Health Research
Research Triangle Park, NC
Contact: Delorise Williams
(919) 558-1200

March 28
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"IHC Cross-Reactivity and Detection Systems for Animal Tissue"**
Speaker: Elizabeth Chlipala, HTL(ASCP), QIHC
Premier Laboratory
University of Colorado
Boulder, CO

April
Histology Society of Ohio State Meeting
Site: Columbus, OH
Contact: Kelly Ferguson
Email: kelly.ferguson@uhhs.com

April 14
Nebraska Society for Histotechnology
Site: Lincoln, NE
Contacts: Konnie Zeitner, kzeitner@nebraskamed.com
Janice Mahoney, jmahoney@alegent.org

April 20
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Fixation (Or How It Is Supposed To Be Done)"**
Speaker: Lamar Jones, BS, HT(ASCP)
Manager, Anatomic Pathology
Wake Forest University
Baptist Medical Center
Winston-Salem, NC

April 21
Texas Society for Histotechnology
Site: Houston, TX
Contact: Judy McKinney
Email: jwebb01@jpshealth.org

April 25
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"Introduction to Polymerase Chain Reaction (PCR) Assays With Applications to Solid Tumor Analysis"**
Speaker: Susan Hunter, SI(ASCP)MP
William Beaumont Hospital
Royal Oak, MI

April 25–27
Tri-State Symposium: Iowa, Minnesota & Wisconsin
Site: West Des Moines Marriott, Des Moines, IA
Contacts: Judi Stasko, jstasko@nadc.wsda.gov
Colleen Forster, cforster@umn.edu
Maureen Decorah, decorah@rarc.wisc.edu

May 4–5
Michigan Society for Histotechnology
Site: McCamly Place Hotel, Battle Creek, MI
Contact: Rachel Kropf
(269) 544-5708
Email: rkropf@rallansci.com

May 17–18
Illinois Society for Histotechnology
Site: The Chateau, Bloomington, IL
Contact: Maureen Doran
(618) 453-1584
Email: mdoran@siumed.edu

May 17–20
California Society for Histotechnology
Site: San Mateo Marriott, San Mateo, CA
Contact: Shirley Chu
(510) 675-6260
Email: chu_shirley_s@yahoo.com

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Educational Opportunities in 2007

May 18
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Immunohistochemistry Mathematics in the Laboratory"**
Speaker: Joel Martinez, BS
Senior Account Manager
BIOCARE Medical
Houston, TX

May 23
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"Implementing Six Sigma, A Case Study"**
Speakers: Bill DeSalvo, HTL(ASCP) and
Kathleen Davis, HT(ASCP)HTL
Sonora Quest Laboratories
Tempe, AZ

June
Tennessee Society for Histotechnology Meeting
Contact: Jennifer Hofecker
Email: jhofecker@yahoo.com

June
Georgia Society for Histotechnology
Contact: Mike Ayers
Email: mayers@newnanhospital.org

June 15
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Apoptosis: A Detection of Cell Death"**
Speaker: Frances Swain, HT(ASCP), AAS Department of Orthopaedic Research University of Arkansas
Little Rock, AR

June 22
ASCP Teleconference
12:00 noon Central Time (800) 267-2727
Title: **"Where Do We Begin? Working Up a New Antibody in Tissue Sections"**
Speaker: Mary Vaughn, HT(ASCP)
Roswell Park Cancer Institute
Buffalo, NY

June 27
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"New Shipping Regulations for Chemicals and Biohazards"**
Speaker: Linda Durbin,
Exakt Technologies, Inc.
Oklahoma City, OK

July 20
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Lab Space — The Final Frontier"**
Speaker: Elizabeth A. Sheppard, MBA, HT(ASCP)
Product Manager
Ventana Medical Systems, Inc.
Tucson, AZ

July 25
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"IHC for Viruses and Other Microorganisms"**
Speakers: Sheron Lear, HT(ASCP)HTL, QIHC and
Alvin Martin, MD
Univ. of Louisville and Brown Cancer Center
Louisville, KY

August 17
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Phosphorylation Pathways and Immunohistochemistry"**
Speaker: Anne Lewin, BS, HT(ASCP), QIHC(ASCP)
Histotechnologist/Research Scientist in Oncology
Bristol Myers Squibb
Princeton, NJ

August 22
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"Ergonomics for Laboratory Professionals"**
Speaker: Jan Minshew, HT(ASCP)HTL
Leica Microsystems, Inc.
Bannockburn, IL

September 7–8
Histotechnology Society of Delaware Fall Meeting
Site: Newark, Delaware
Contact: Michele Hart
(302)733-3657
Email: mhart@christianacare.org

September 21
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Normal and Abnormal GI Tissues: A Comparison of Mucosal Morphology and Staining Characteristics"**
Speaker: Pamela Colony, PhD, HT(ASCP)
Program Director of Histotechnology
State University of New York
Cobleskill, NY

September 26
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"Muscle Anatomy, Special Stains and Enzyme Histochemistry"**
Speaker: D. Wilson, MD
William Beaumont Hospital
Royal Oak, MI

October 19
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"The Biology of Cancer"**
Speaker: Jerry Santiago, BS, HTL(ASCP), QIHC
Pathology Technologist
Shands Hospital
Jacksonville, FL

October 26–31
NATIONAL SOCIETY FOR HISTOTECHNOLOGY SYMPOSIUM/CONVENTION
Site: Denver, Colorado
Contact: Aubrey Wanner
(301) 262-6221
Email: Aubrey@nsh.org

November 16
University of Texas Health Sciences Ctr/San Antonio
Teleconference 12:00 pm Central Time (800) 982-8868
Title: **"Instrumentation — A Capital Idea!!!"**
Speaker: Terry Braud, HT(ASCP)
Anatomic Pathology Supervisor
Holy Redeemer Hospital Laboratory Meadowbrook, PA

November 28
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"Fixation — From Acetone to Zinc"**
Speaker: Peggy Wenk, HTL(ASCP)SLS
William Beaumont Hospital
Royal Oak, MI

December 19
NSH Teleconference 1:00 pm Eastern Time
Contact: (301) 262-6221
histo@nsh.org
Title: **"Water Quality for the Histology Laboratory"**
Speaker: Ethel Macrea, HT(ASCP), QIHC
Southwest Skin Pathology Service
Tucson, AZ

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