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Dazzling Color in the Land of the Inca: A Centuries-old Dye Still Important in Histology Today

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As a child, I was exposed to the culture of the Andeans. More than anything in this world, I wanted to meet the young Peruvian girl with her llama who was pictured on a postcard given to me by a family friend who shared his travel stories with my family and always returned from his adventures with photographs and trinkets. From Peru he brought a caiman (a four-legged reptile from the alligator family), prickly cacti, and colorful textiles. I still have memories of my grandmother warning me and my siblings to avoid playing near the cactus growing at the side of her house, never imagining how that cactus might some day become connected to the work I would eventually do in my professional career.

Fast-forward 40 years to the summer of 2006 when an opportunity to study abroad in Peru became available to me, which I accepted without hesitation. Sixteen glorious days in Cusco, Peru, the “navel of the universe” as it is referred to by the native people there, sounded like an opportunity to fulfill my dream. Prerequisite details included taking survival Spanish and a crash course in the Andean culture, and becoming familiar with the native language of Quechua. Also, I had to select a course of study. I knew immediately I wanted to study the cochineal beetle because I wanted to see the origin of the bright red dye in the colorful Peruvian textiles from my childhood, which also happens to be the same dye used in many histology laboratories for staining carbohydrates (Fig. 1).

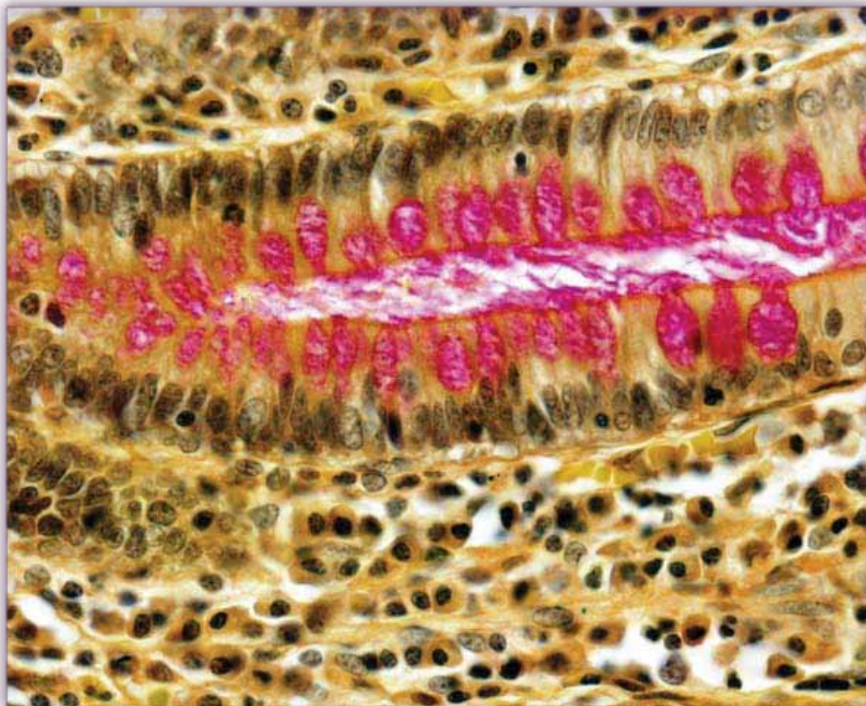


Fig. 1. Colonic mucosa illustrating intense staining of goblet cells. The intense pink color is from the cochineal extract, carmine. Mayer's mucicarmine, 400X.

IN THIS ISSUE

Dazzling Color in the Land of the Inca: A Centuries-old Dye Still Important in Histology Today . . 21

Reorientation of Tissue Microarray Cores for Volumetric Examination of Tissue Specimens 25

Dewaxing Histology Slides Using Common Household Products: Let Your Lab Sparkle Green! 28

A Retrospective Review of the Advantages of Glycol Methacrylate (GMA) as an Embedding Resin . . 30

ID Gram Stain: A Modified Brown-Hopps Method for Gram-positive and Gram-negative Bacteria . . . 36

Unusual Exogenous Pigmentation in Frontal Gingiva: A Brief Case Report 40

Mark Your Calendar! 42

Cochineal, also known as carmine, is one of the reddest dyes the natural world has ever produced. It has been used on the robes of kings and cardinals, on the lips of screen goddesses, on the camel bags of nomads, and on the canvases of great artists.¹ A common staple in histology laboratories for the staining of carbohydrates, the rich origin of this dye is little known by many who obtain it from scientific supply houses for staining tissues. How fitting that a color that has had such a widespread impact on both the Old World and New World would play such a significant role in the microscopic world as well.

My first glimpse of the Andes was from the window seat of a small plane. Majestic mountains appeared as islands in a sea of clouds, accompanied by a brilliant sunrise. As we approached Cusco, the snow-covered peaks, volcanoes, and terraced highlands appeared below the cloud cover. After touching down on a small landing strip, vendors immediately appeared with their wares and coca leaves. We were advised that coca tea is highly recommended to avoid altitude sickness in the first few hours after arrival in Cusco, which is approximately 12,000 feet above sea level.

Cusco was once the capital of the Inca Empire and arriving there began my journey back in time. At the height of its power (from about 1400-1532), the Inca Empire controlled 10,000 kilometers of roads. Without the use of wheels and horses (let alone telephones and emails), government communication was accomplished using a relay team of messengers who would sprint for 20 kilometers at a time to deliver a message. The people had no written language, and when the message was too complicated for a messenger to remember, he carried color-coded cords, or *quipus* (from "khipu," the Quechuan word meaning knot) to pass on the information (Fig. 2). Every color and knot had a meaning associated with it.^{1,2} Cochineal dye, prized for its ruby red color, was one of the colors used in the strings of the quipus. The dye was also used by women for blush, by potters for decoration, and by artists in frescoes. Most of all, it was found in the textiles of the region. But in the quipus (sometimes referred to as "talking knots"), a red-knotted cord represented life, power, and death for the Inca, all in a single piece of string.

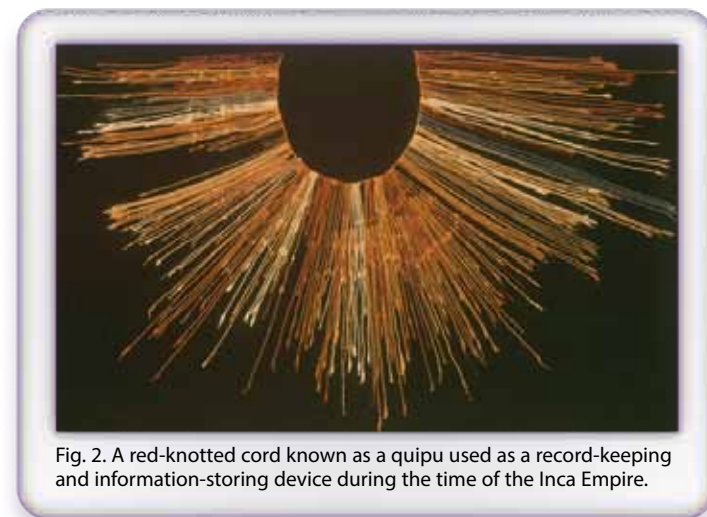


Fig. 2. A red-knotted cord known as a quipu used as a record-keeping and information-storing device during the time of the Inca Empire.

Weaving of fibers was so important to Incan society that the Inca would conquer surrounding communities and bring skilled artisans and weavers back to Cusco. The most prized possessions offered to Spanish conquistadors were fibers and colorfully dyed textiles. The process and tradition involved in making quality

Peruvian textiles are highly complex and special. It would take months to weave one of these colorful fabrics (Fig. 3), often using camelid fibers from llama, alpaca, and vicuña. Incan textiles were considered a necessity because of unpredictable and extreme weather conditions in Peru (Fig. 4). Along with their functional value, textiles were also indicative of the wearer's status and role in society. Because of the considerable talent and skill required in creating them, textiles were also considered works of art, much like the art and science of histotechnology. There is actually a common link between the textile art of Old World Peru and histology, and my studies in Cusco would allow me to explore that common link—the dye produced by the cochineal beetle.



Fig. 3. Colorful handmade garments and textiles are readily available for purchase by visitors to Cusco, Peru.



Fig. 4. Peruvian-made garments are not only beautiful and colorful, but functional and necessary in this climate.

The earliest use of dyes in microscopy is believed to date back to the times of Robert Hooke (1665) and Anton van Leeuwenhoek (1719). Although reported to have been used in the 1700s, cochineal was prized among biologists throughout the mid to late 1800s, having been referenced in countless scientific publications of the time.^{3,4} This is best illustrated in *The Microtome's Vade-Mecum* from 1885 where cochineal and carmine are the main topic of discussion in 6 of the chapters.⁵ Of the dyes used in early histology, none was so highly prized as carmine. Even today, biologists would be loathe to part with it.³ Typically used in alcohol solutions containing various acids, bases, and mordants, and sometimes combined with indigo (indigo-carmine), carmine was best studied and described in 1892 by Paul Mayer, whose legacy to the histology

community is the Mayer's mucicarmine stain, which is still performed in modern clinical histology laboratories. In biology applications, the dye is often combined with alum mordant to achieve fastness and staining specificity. Colors from pastel pink to deep purple can be achieved in textiles when the dye is combined in different recipes with various metal salts.

The deep crimson cochineal dye is extracted only from female cochineal insects (*Dactylopius coccus*, a scale insect). They are parasites found on the *Opuntia* cactus where they feed on plant moisture and nutrients (Fig. 5). Cochineal extract is used to produce scarlet, orange, and other reddish tints. The coloring comes from carminic acid found in the insect's shell. Cochineal extract's natural carminic acid content is usually around 19% to 22%. It takes about 80,000 to 100,000 insects to make 1 kilogram of cochineal dye.⁶ Peru is the largest exporter of cochineal today, exporting about 200 tons annually.



Fig. 5. The powdery white areas on this cactus are the cochineal insect. Only the females yield the dye.

Unknown to many, cochineal extract is widely used in many modern consumer products, including foods and beverages, pharmaceuticals, and cosmetics including hair care and skin care products, lipstick, face powder, rouge, and blush. And cochineal is still used in textiles today. If you wonder whether you are ingesting insect extract, look for terms such as Cochineal, Cochineal Extract, Carmine, Crimson Lake, Natural Red 4, C.I. 75470, E120, and even some ingredients listed as "natural colorings" on the labels of your favorite foods.^{7,8}

In the *Encyclopedia of Microscopic Stains*, the following histologic carmine-based stains are listed: carmalum, paracarmine, acetocarmine, alum carmine, ammonia carmine, Best's carmine, borax carmine, iron acetocarmine, lithium carmine, mucicarmine, and picrocarmine variants.⁹ As with other natural dyes, the challenges of working with carmine stem from fluctuations in purity that can vary depending on the source from which the dye is obtained. Some propose that carminic acid, the active ingredient in cochineal dye, may be used as an alternative in the laboratory in order to achieve greater consistency from batch to batch, although it may not be as readily available as carmine or cochineal extract from commercial sources. In practice, once you have obtained a good batch of carmine, it is an invaluable asset to the laboratory.

Though it is possible to analyze commercial extract for available carminic acid content, Horobin and Bancroft state that it is not worth the trouble; they recommend that you try alternative suppliers and only buy small amounts until you find a good supply.¹⁰

Today, Best's carmine method for staining glycogen and Southgate's modification of Mayer's mucicarmine stain for mucin¹¹ are probably among the best-known applications of carmine in the clinical histology laboratory. Mucicarmine is perhaps most useful as a simple screening tool for distinguishing mucin-negative undifferentiated squamous lesions from mucin-positive adenocarcinomas. The likely mechanism of action for mucicarmine is the binding of the cationic metal complex dye to the anionic glycosaminoglycans present in mucins. The active species is probably a cationic 2:1 carminic acid-aluminum chelate¹⁰ (Fig. 6). Mucicarmine can also be used to stain the mucopolysaccharide capsule of *Cryptococcus neoformans* (Fig. 7).

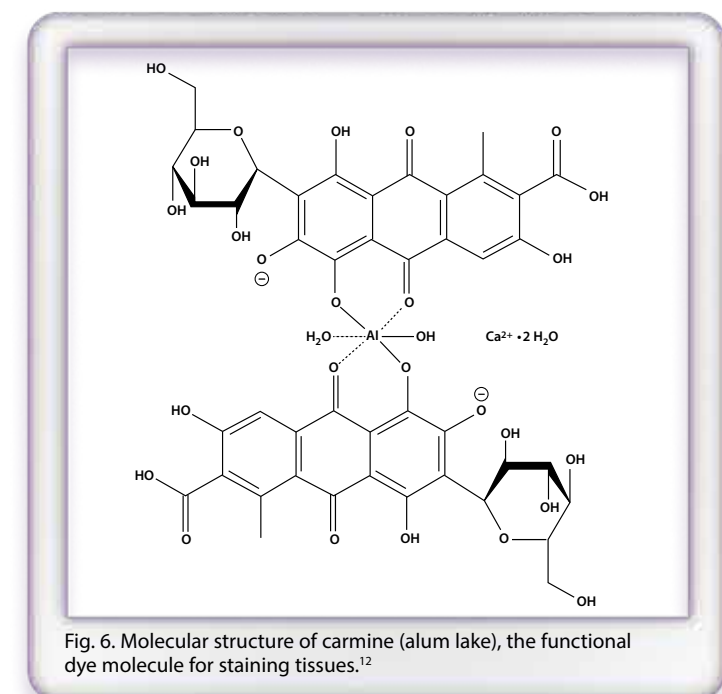


Fig. 6. Molecular structure of carmine (alum lake), the functional dye molecule for staining tissues.¹²

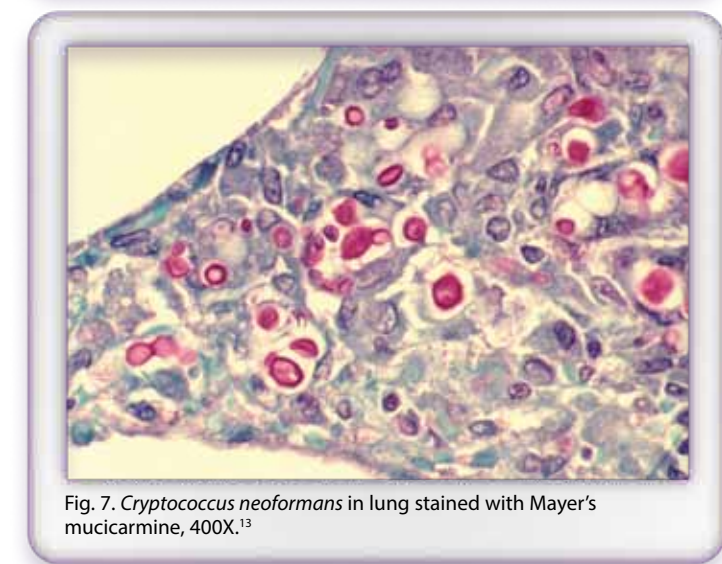


Fig. 7. *Cryptococcus neoformans* in lung stained with Mayer's mucicarmine, 400X.¹³

Best's glycogen method has largely been supplanted by the periodic acid-Schiff (PAS) method, although it has been suggested that oxidizing in periodic acid will increase the specificity of Best's technique. Carmine is the aluminum or calcium-aluminum lake of carminic acid and is only slightly soluble in water. The addition of an acid or base will increase its solubility and staining ability.

While in Peru, I would spend days hiking the Inca trail, continuing my search for the cochineal beetle as I ventured through the many ruins and villages by horse, raft, and on foot. It was one such day that I found myself clinging to a terraced ledge along the trail. There, as the sun glistened, I saw the telltale white powder on a cactus within my reach. Carefully I swiped a small "seed" and crushed the beetle between my fingers. The beautiful juice stained my fingers and in my excitement I forgot to photograph the result. It was a private moment in which I reflected on my introduction to cochineal, which was on the cover of Sheehan and Hrapchak's prized textbook *Theory and Practice of Histotechnology*.¹¹

The following day I would finally meet the women from the village of Chinchero, known as the "town of the rainbow." As we got off the bus, children greeted us. I had come prepared with a backpack filled with pencils, sharpeners, and candy. The children received my gifts graciously and led us into the town center. Several women were preparing textile fibers with boiling water. Our guide explained that a root known as saqta would be added to clean the wool. She also explained that the women used this root to clean their hair and make it soft and shiny. I inquired about cochineal and immediately the women shared their dyes, showing me the various mordants of alum, lemon, lime, and volcanic rock (Fig. 8.) Knowing this was the hometown village of Nilda Callanaupa, the director of the Center for Traditional Textiles of Cusco, I thought this would be the perfect place to



Fig. 8. A vendor dozes while waiting for a customer to buy her vivid dyes.

find a "manta," or shawl, so I headed to the center of town. My eyes were drawn to a specific manta that happened to match the copper backsplash of my kitchen at home. I bargained with the seller and wrapped myself in my new artwork on the ride back to Cusco. The Center's goal is to preserve the art of the complex Peruvian weavings that were previously handed down from generation to generation by word of mouth because they were not recorded in any permanent way (Fig. 9). More than 44 complex weave patterns dating back to 500 AD have been identified and recorded for future generations.



Fig. 9. Carolina, right, a local weaver from the Center for Traditional Textiles of Cusco, demonstrates a double loom.

During my search for the cochineal beetle, it occurred to me that our reliance in histology on a dye from an otherwise little-known insect parallels its importance to generations of Peruvians since the time of the Inca. This beetle and the vivid red color it provides has been central to Andean culture as a form of art and communication for hundreds of years. Centuries later, this same insect plays a pivotal role in revealing the microscopic complexities of tissues. Although many histotechs are unaware of the unique origin and history of cochineal dye, it is fascinating to me that a single species of insect could be so important to both ancient and modern societies, and remains so even today.

Note: The indigenous people of central Peru continue to struggle to this day. Their economy and ability to feed their families center around the Peruvian textile industry and the harvesting of cochineal. There is no synthetic alternative for this dye. Natural dyes such as cochineal have played an essential role in the evolution of microscopy and the discipline we call histotechnology today.

Visit the Center for Traditional Textiles of Cusco's website at <http://www.textilescusco.org/eng/index.html> and support the art of textiles and natural dyes.

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Reorientation of Tissue Microarray Cores for Volumetric Examination of Tissue Specimens

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Abstract

In this report we describe a technique for arranging tissue cores obtained using the Tissue-Tek® Quick-Ray™ Tissue Microarray System (Sakura Finetek, USA, Inc., Torrance, CA) in a manner that allows a full-thickness examination of the tissues under study. This method is simple and efficient to perform. It gives information on the morphologic topography of tumors and it can help track special cellular structures throughout the entire specimen thickness.

Introduction

In histology and histopathology, analysis of two-dimensional microstructure does not generate the necessary observations to form well-grounded insights into biological principles. Examination of consecutive sections obtained by either serial sectioning or step sectioning may be needed to study the track of some structures or to find the extent of a lesion. In *serial sectioning*, sections are collected from the very first cut that includes any tissue. Ribbons of 10 (1-10, 11-20, 21-30, etc) are picked up and mounted on the slides. In *step sectioning*, an alternative to serial sectioning, sections are taken at specified intervals through the block. The request is made for every nth section for a total of "n" sections, where intervening sections or sections on either side of each stained step section may also be collected. Occasionally, both serial sectioning and step sectioning may be required for a sample^{1,2}; both are time-consuming and require great care in collecting and mounting the required tissue sections.

Variations in the proportion of surface occupied by tumor (percentage carcinoma), carcinoma volume, histological grade, capsular penetration, and margin positivity are assessed in a completely sampled organ. Tumor grading includes an assessment of proliferate potential by counting mitotic figures per microscopic field. Organs are embedded and then each block may be serially sectioned. The morphologic prognosticators of tumor parameters—volume, histological grade, margin positivity, and pathologic stage—may be altered by complete serial sectioning,³ otherwise nodal metastasis may go undetected.

Since its development in 1998 by Kononen et al,⁴ tissue microarray (TMA) has become a useful tool for research. Potential applications of microarray technology in diagnostic histopathology are also of great benefit. The aim of the present work was to use this technique as a substitute for serial and step sectioning in order to give more detailed information on examined tissue specimens in the volumetric configuration.

Materials and Methods

Postmortem tissue 4 to 7 mm thick was fixed in formalin, dehydrated, and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E) by conventional method. Surface sectioning in the first stage of preparation was needed to study the track of certain structures in a two-dimensional presentation (x and y axes).

In the second stage of preparation, prepared slides were examined microscopically, and areas of interest were identified. To obtain the tissue cores, we used the 2 mm punch tip from the Tissue-Tek® Quick-Ray™ Tissue Microarray System (Fig. 1) to remove 5 cores from paraffin-embedded tissue (donor block) as illustrated in Fig. 2. The extracted tissue cores were arranged in parallel format and laid down horizontally with forceps. The array platform with the tissue cores in situ was then transferred to the stainless steel mold (Fig. 3).

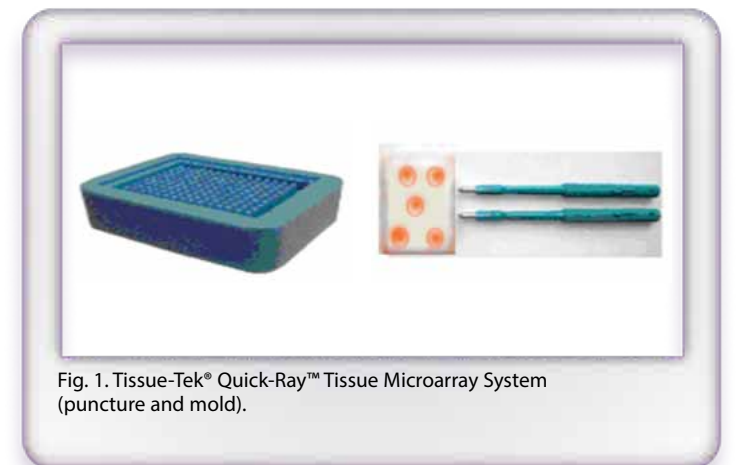


Fig. 1. Tissue-Tek® Quick-Ray™ Tissue Microarray System (puncture and mold).

Melted paraffin was gently poured into the mold from the sides (recipient block). To ensure gradual and uniform setting of the block, the mold was initially cooled at room temperature and then transferred to a refrigerator for 20 minutes before sectioning. From these blocks, 5 to 7 micron sections were cut on a rotary microtome and stained by routine H&E staining. We could thus prepare 50 to 100 sections from a single set of tissue cores.

Results

Typically, each tissue section is 2 to 5 mm long and 0.2 mm thick. Images of one of these cores were sized to cover the microscope low power (4X) objective (Fig. 4). Scanned images of successive sections were then stacked to obtain the volumetric data set. Samples consisted of cylindrical cores to ensure a uniform sharp cutting edge.

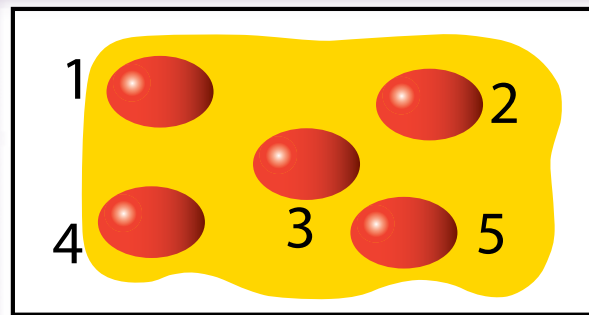


Fig. 2. Diagram showing circular areas of punctured cores from the donor block using the TMA method.

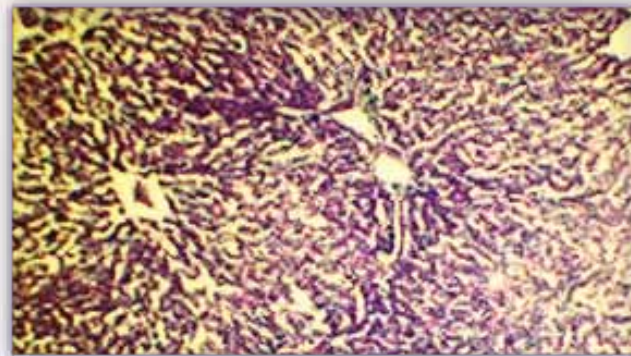


Fig. 4. Section of tissue core (0.2 mm thick x 5.0 mm long) from liver. H&E, 40X.

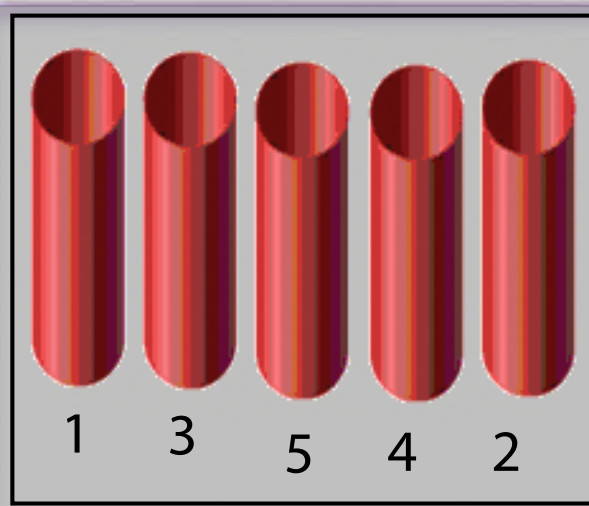


Fig. 3. Diagram showing the arrangement of the cores in parallel format in the stainless steel mold.

This method has important clinical implications that should be considered. Proliferative kinetics (the rate of tissue growth) is important in understanding the growth pattern of tumors and fibrosis. Mitotic index counting is a measure of cellular proliferative activity in histologic sections.⁵ The presence of a tumor and its distance from the surgical margin is an essential element of tumor assessment.⁶ Moreover, the mitotic index is also useful for the evaluation of cytotoxicity, drug interaction, and therapeutic decision making.⁷

Three-dimensional histopathology, as discussed in this report, offers additional value when assessing surgical margins and mitotic activity in tumor samples. For this purpose, it is recommended that duplicate tissue cores from different regions of donor tumor blocks be taken—one set for sectioning by conventional method and the other set for reorientation as described above. The collective data will be a better indication for screening and evaluation of changes in the rate of proliferation of tissue (three-dimensional screening).

Our experience with this novel technique indicates that this method is both easy to perform and cost-effective, and it offers great potential for laboratories with minimal technical sophistication and financial limitations.

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Discussion

Conventional serial sectioning restricts the maximum observable thickness of the tissue in the x and y axes, therefore limiting z-axis resolution. The method we described here allows three-dimensional reconstruction by first aligning and then stacking the images from consecutive planes, which allows volumetric image data to be constructed.

In tumors, serial sectioning may reveal metastases that might otherwise go undetected. In neurobiology, for example, there is a need to relate cellular structure to organization of neural circuits. Analysis of three-dimensional reconstruction of even one to a few cells is time-consuming and still does not generate the necessary number of observations to form well-grounded insights into biological principles.² Sections from tomography of reoriented tissue microarrays also have other advantages including quantitative reliability, detection sensitivity, scalable volume field of view, and direct applicability of tissues.



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Dewaxing Histology Slides Using Common Household Products: Let Your Lab Sparkle Green!

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Introduction

I live on the island of St. Thomas, one of the four US Virgin Islands—America’s paradise. Hues of blue surround our tiny island. Palm trees sway in the warm breeze. A fresh Caribbean lobster sizzles on the grill. Imagine as reggae music swirls from a not-so-distant party on the beach as you hold a cool rum punch in your hand. You glide side to side in your hammock, no worries, just paradise! I’m betting that cancer-causing toxic chemicals never entered your thoughts as you imagined that tropical description, right? But can there be such a thing as an histology lab without dreaded toxic chemicals?

Dr. Francisco J. Landron, Chief of Pathology & Laboratory Services at Schneider Regional Medical Center in St. Thomas, had just returned to work after taking a few months off. Unfortunately, he had not been out on the golf course; he had been battling for his life. A diagnosis of bilateral renal cell carcinoma sent shockwaves through our whole medical community. Triumphant in his battle and determined to make sweeping improvements in our working environment, Dr. Landron, his wife, and his oncologist had one simple request of me as the histology laboratory supervisor: rid the department of chemicals! With the 3 words “Let’s Go Green,” I was in for the challenge of my career. Going green in a histology lab seemed like an impossible task. How do you prepare tissues without chemicals?

Then I remembered a quote attributed to Christopher Columbus that my grandfather taught me:

“You can never cross the ocean unless you have the courage to lose sight of the shore.”

I read an article once about how the word *courage* should be incorporated into the vision of an organization. I believe this wholeheartedly now. I decided the moment Dr. Landron pulled me into his office for his “Let’s Go Green in Histology” meeting

that I would be fearless and have the courage to lead innovation and not merely follow the standard practices used in most histology laboratories.

I had just received my copy of *Microtime*, a newsletter from the Georgia Society for Histotechnology, which contained an article written by René Buesa entitled “Histology Without Xylene,”¹ which got me started. Mr. Buesa was easy to contact via email and extremely helpful in the troubleshooting that followed. He is also a constant source of information on Histonet, an online histology forum.

Materials and Methods

Autopsy tissue was used to develop and validate a dewaxing method through trial and error. A laboratory water bath, a variety of dishwashing detergents, and a timer were used to evaluate the variables. With all the possible variations, it required several weeks to run the trials. I was fortunate to have the assistance of a summer intern from the Virgin Islands Department of Labor-sponsored LIFT program who committed to completing our trials before her summer internship ended.

After establishing optimum incubation times and temperature, I noticed staining voids in fatty and dense tissues. This led me to research the concept of surface tension in water. I read an article that discussed similar principles used in automatic dishwashers, which is basically the same concept of removing waste material using hot water and soap. These devices use “rinse agents” to lower the surface tension of the water. This information prompted me to try using a rinse agent in our experiments. The rinse agent was able to eliminate the staining voids we had experienced earlier. The following is the procedure derived from those experiments that we have been using for dewaxing slides since July 2011.

Dewaxing Histology Slides

A water bath is utilized for this procedure. The temperature is critical—it must be in the narrow range of 90°C to 95°C. Heat-resistant staining dishes are heated with solutions at least 1 hour before use to achieve the required temperature. This method will not work if the temperature of the solution is less than 90°C.

1. Place tissue sections on Super Frost Plus™ slides (Erie Scientific Co., Portsmouth, NH) to ensure good section adhesion.
2. Slides must be totally water free before drying in an oven at 65°C for at least 30 minutes.
3. Immediately place the dried sections (batch size 20 slides maximum) in 2.0% dishwashing liquid solution at 90°C to 95°C for 1 minute. Repeat this step for a total of 3 detergent incubations. Agitate the slides gently. **Note:** Do not agitate enough to make suds form.
4. Wash the slides in rinse agent solution for 3 changes at 30 seconds each.
5. Wash the slides in running warm tap water for 3 minutes or until water is clear.
6. Wash the slides in sterile or deionized water.

Soap Solution

Sterile or deionized water.....250 mL
Dawn® dishwashing liquid4.5 mL

Rinse Agent Solution

Tap water250 mL
Cascade Rinse Agent®3.0 mL

Our H&E staining protocol is performed on an automatic staining instrument.

Automated H&E Staining Protocol

STEP	FUNCTION	TIME
1	Room temperature sterile or deionized water	1 minute
2	Room temperature sterile or deionized water	1 minute
3	Room temperature sterile or deionized water	1 minute
4	Hematoxylin	8 minutes
5	Tap water rinse	1 minute
6	Tap water rinse	1 minute
7	Tap water rinse	1 minute
8	Clarifier	30 seconds
9	Tap water rinse	1 minute
10	Tap water rinse	1 minute
11	Tap water rinse	1 minute
12	Eosin-Y	5 minutes
13	Sterile or deionized water	1 minute
14	Empty (mix)	
15	Empty (mix)	
16	Empty (mix)	
17	Empty (mix)	
18	Empty (mix)	
19	Oven	7 minutes
20	Coverslip	

Steps 14-18 allow the machine to shake off the excess water on the slides. The containers in those steps are empty and the mechanical arm is set to “mix,” which is a simple up-and-down movement. Contrary to what may be done in other laboratories, we allow the stained slides to fully dry before coverslipping, which eliminates any need for alcohol or clearing agents in the staining protocol.

Dawn and Cascade Rinse Agent are registered trademarks of Procter & Gamble Company.

Discussion

With the introduction of heat at the end of the staining cycle, we have eliminated the need to dehydrate and clear the slides for coverslipping. We simply coverslip the dried slides with a mounting medium diluted with the same clearing agent we used for tissue processing. Optimum results are achieved when the mounting medium has the consistency of mineral oil.

We did an extensive blind side-by-side comparison study. The lab passed The Joint Commission and Centers for Medicare and Medicaid Services surveys with no recommendations.

We have realized an incredible savings since using this new dewaxing procedure. With the old method, it cost \$81.03 for every 100 slides for a clearing agent, alcohol, and bluing reagent. As you can see from the Table below, that cost was eliminated with the new method.

Reagents	Old Method	Cost/100 Slides	New Method	Cost/Week
Clear-Rite 3®	7,700 mL=2.03 gal	\$35.24	Eliminated	\$00.00
Reagent Alcohol	9,100 mL=2.44 gal	\$40.58	Eliminated	\$00.00
Bluing Reagent	700 mL=0.19 gal	\$5.21	Eliminated	\$00.00
TOTAL		\$81.03		\$00.00

All reagents listed above are available from Thermo Fisher Scientific, Waltham, MA; however, there is no need for those reagents in the method reported here.

- Our new H&E staining method utilizes Dawn dishwashing liquid (\$10.21 for a 6-month supply), which reduced our costs to \$0.04/100 slides
- Clear-Rite 3, bluing reagent, and reagent alcohol are not approved by the EPA to be flushed down drains. As a result, we had been spending \$18.64/gallon each month (\$223.68/gallon each year) to dispose of these chemicals. With this new procedure, we’ve reduced our cost to \$0 for disposal!

Conclusion

I have shared this procedure with many histologists around the world. In writing this article, I hope that many more histologists can benefit from our trials and become like-minded in the move to make their histology labs “Sparkle Green”!

I am not a research scientist nor do I claim to know everything about histology, but I do try to learn something new every day. The quotation attributed to Christopher Columbus that my grandfather shared with me is a reminder that we have to be willing to take a risk to achieve progress. Trial after trial, failure after failure, success after success, I intend to persist until I feel comfortable with stepping back on the shore, which may never happen because I have become accustomed to these waters!

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A Retrospective Review of the Advantages of Glycol Methacrylate (GMA) as an Embedding Resin

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Introduction

In the mid 1970s, pathologists and researchers, in their efforts to achieve superior nuclear detail in light microscopy, were turning to resin embedding techniques. One such polymer that gained significant investigative interest was glycol methacrylate (GMA). GMA was prized for its outstanding nuclear detail, versatility, and relative ease of use, especially compared to methyl methacrylate (MMA).¹ This afforded the pathologist/investigator an alternative for the study of mineralized materials such as undecalcified bone. Touted as a water-soluble resin, it was initially speculated that GMA could be removed from the tissue and that staining could easily be performed “through the plastic.”

As it became more popular, GMA became available in kit form from various sources, including the JB-4® Embedding Kit (Polysciences, Inc., Warrington, PA), which made working with the material even easier. Specialized block holders and molds specific for GMA embedding were developed. Retracting microtomes, such as the Sorvall JB-4 and Hacker-Bright, were finding their way onto laboratory benches, formerly the exclusive domain of rotary paraffin microtomes. Two different styles of glass knives allowed pathologists to achieve 1.5 to 2 micron thick sections from samples of various sizes. Forward-thinking pathologists of the day began “putting aside a piece for plastic” at the grossing table.

Although GMA embedding offered significant advantages in obtaining thinner sections than could be achieved in paraffin, it struggled to gain uniform acceptance. Pathologists unaccustomed to the exceptional cellular detail afforded by GMA expressed that they “liked the old look (of paraffin sections) better” and felt more assured of their interpretations with paraffin. In our laboratory, those pathologists working with bone and skin favored the thin plastic sections achieved with GMA, while those specializing in kidney and liver pathology preferred paraffin sections. Another common complaint with GMA sections was that the hematoxylin and eosin (H&E) stain was too light. Of course it looked light—the section was half as thick as a conventional paraffin section!

Staining challenges could be overcome if staining time was doubled, which often resulted in darker staining, but at the

expense of nuclear clarity. Substituting Gill’s hematoxylin for other hematoxylin formulations overcame the light staining somewhat, but not entirely. While we succeeded in adapting a number of special stains to GMA sections, not all stains could be carried out successfully in this medium. As a rule, we increased the concentration of dyes where possible, increased the staining time (in some cases tripling it), and decreased differentiation times, all of which provided a good starting point for converting a paraffin staining procedure to GMA.

Around the same time that researchers were trying to optimize GMA embedding, immunohistochemistry was first introduced. However, immunohistochemistry was difficult to perform on fresh frozen sections, so the concept of converting those techniques to formalin-fixed, paraffin-embedded (FFPE) tissues seemed futuristic. GMA never gained traction as a routine embedding medium and it just seemed to fade away.

Renewed interest in resin embedding for light microscopy has occurred in recent years, driven by broad-based research in biomaterials. The Hard Tissue Committee of the National Society for Histotechnology (NSH) has experienced a resurgence of inquiries pertaining to resin applications in bone histology, especially crossover applications between paraffin and resin embedment for bone histology.

Materials and Methods

The Bone and Muscle Laboratory of the State University of New York at Stony Brook in the early 1980s had the interest and the available resources to test the applications and practicality of GMA embedding using the JB-4 system. All necessary components from the consumables kit—the mold/object holder combo, the JB-4 retracting microtome, and LKB triangular glass knife breaker—were incorporated in our early investigations. We initially adhered to the manufacturer’s instructions,² but over time we developed modifications that served us well in our continuing efforts to optimize. For instance, while the instructions stated that the specimens could be placed in catalyzed monomer directly from 95% ethanol, we eventually ran all of our samples to absolute ethanol, which yielded more reliable GMA infiltration.

Over time we discovered that the standard ratio of 0.9 g of catalyst per 100 mL of monomer did not have to be so precisely measured for infiltration purposes, which meant that “lot overruns” could be combined and stretched to get even more utility from the components. We did not hesitate to use catalyzed monomer made up to 3 weeks prior for infiltration, but for the actual polymerization of the specimen we recognized that the most consistent polymerization was derived from precise measurements with freshly combined components. We never made more than a 100 mL lot of Polymerization Catalyzed Solution A and never used it for polymerization if it was more than 5 days old. Additionally, we came to recognize that although a 25:1 ratio of Catalyzed Solution A to activator (Solution B) was touted as the norm, we found that a 40:1 ratio produced a superior block suitable for a variety of tissues including skin and bone.

Although precision was important for consistent results, the manipulation of this polymerization monomer was crucial. While the infiltration monomer could be left at room temperature for stretches of time during the course of the work day, the

polymerization monomer was always used at 4°C. Infiltration was generally carried out in closed containers at ambient pressure. Polymerization generates heat and, if carried out in open air, should be carried out in a chemical fume hood. (The JB-4 kit directions suggest vacuum applications but we found no significant advantage to using vacuum.) The components begin polymerizing as soon as the products are combined. We noticed that, if our 40:1 polymerization batch was actually 40 mL of Catalyzed Solution A and 1 mL of activator (Solution B), the first blocks poured were better than the last blocks. We theorized that during the lag time between the first and last blocks, the polymerization was progressing in a less than uniform manner. Our remedy for this was to make small batches of catalyzed polymer (as small as 8 mL of Catalyzed Solution A to 0.2 mL of activator [Solution B]), mixing the two portions for the shortest time possible, then distributing the mix immediately while working on a heat-absorbing surface such as the synthetic stone lab counter in the hood. Using aluminum block holders also facilitated heat dispersal compared to plastic holders, which didn’t disperse the heat. The combination of these factors extended the polymerization time significantly and produced the hardest possible blocks, which we considered advantageous.

In order to achieve optimal sections from GMA blocks, a retracting microtome was essential. Standard rotary units of the time would allow subtle contact between the back edge of the knife and the block face on the up stroke. This was not an issue in paraffin sectioning but, when sectioning resins such as GMA as thin as 1.5 microns, this contact led to unsightly scoring and potential tearing of the sections.

The standard steel knives of the day simply did not lend themselves well to optimization of the GMA technology; the best GMA sections were produced using freshly prepared glass knives. Depending on the microtome, the options for glass knives were either a triangular knife or the broad-faced Ralph knife, each of which was made by the histologist with a specialized instrument used to uniformly break the glass strips into the desired configuration. Inconsistencies in knife quality were common and durability was excessively variable, but the results were worth the effort. We initially attempted to conduct studies using standard rotary microtomes to section GMA samples,³ but we eventually realized that consistently superior sections require a retractable microtome. By 1987, the *Journal of Histotechnology* was displaying ads for the LKB Rotary One and the Reichert Jung 2050. Additional technical idiosyncracies were noted and dealt with as applications expanded.⁴⁻⁸

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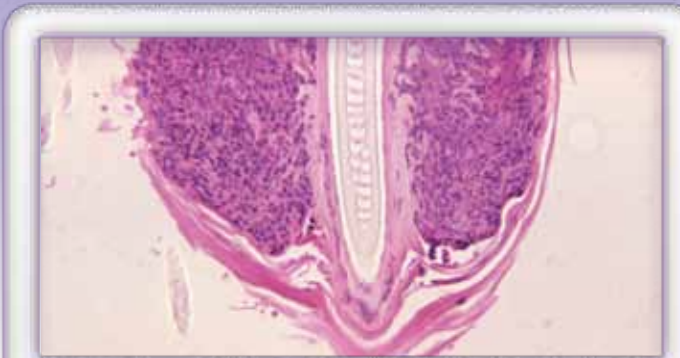


Fig. 1. A 2-micron section of GMA-embedded dermis sectioned longitudinally through an infected hair follicle. H&E staining (Gill's II for 12 min) shows stratified squamous keratinizing epithelium and dermis with a hair follicle. Numerous acute inflammatory cells can be seen in the dermis. 40X

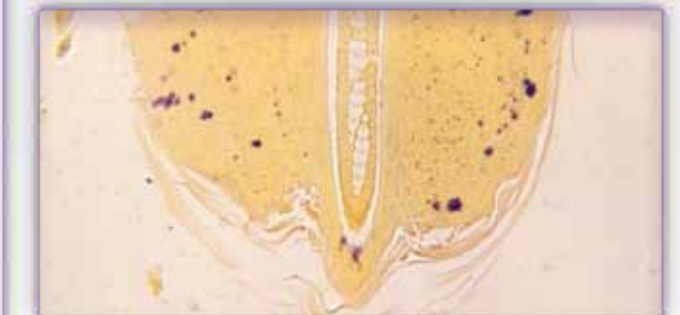


Fig. 2. A 2-micron GMA-embedded serial section of Fig. 1. Modified Gram stain demonstrates groups of gram-positive organisms within the dermis as depicted by the areas of dark-blue staining. 40X

Results

Studies derived from GMA sections eventually went beyond skin (Figs. 1 and 2) and other soft tissues such as lymph nodes, muscles, and nerves (Figs. 3 and 4) to include cultured articular chondrocytes,^{9,10} xenografts of rabbit growth plate chondrocytes,¹¹ and the study of matrix formation in chondrocytes.¹² These mildly mineralized tissues lend themselves well to GMA embedding (Figs. 5, 6, and 7). Additionally, the analysis of osteoid seams in iliac crest wedge biopsies in the diagnosis of osteomalacia widened the scope of our GMA applications (Figs. 8 and 9). Each of these studies benefitted from the fine cellular detail and mineralized definition afforded by GMA embedding. We even sectioned insects such as ticks (Fig. 10) as part of our department's groundbreaking investigation into Lyme disease.¹³ Embedding plastics and films into GMA was attempted, leading to applications for the histologic analysis of biomaterials (Figs. 11 and 12)¹⁴ and the Ilizarov surgical modality for bone defect repair based on the theory of distraction osteogenesis (Figs. 13 and 14).^{15,16}

One of our more interesting clinical applications was the sectioning of bone marrow cores in GMA. During the 1980s, there was an acute appreciation for quality. Our pathologists were demanding textbook-quality slides and they worked closely with the histologists to ensure high-quality outcomes. Our greatest challenge was to achieve a short turnaround time for bone marrow specimens while maintaining high-quality sections. This was truly a time when the state-of-the-art in

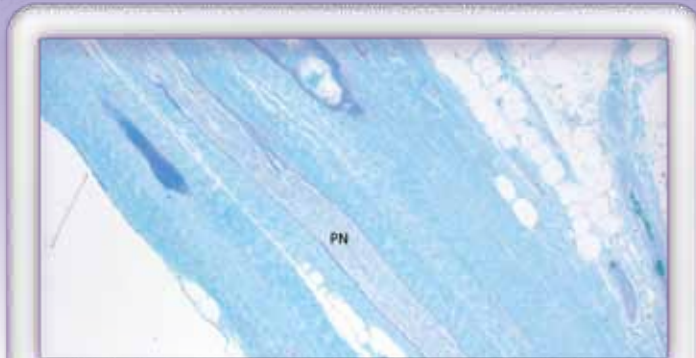


Fig. 3. A 2-micron GMA-embedded longitudinal section of muscle and nerve. Specimen was pinned in 4% paraformaldehyde prior to formalin fixation. Luxol fast blue-periodic acid-Schiff (LFB-PAS) stain shows skeletal muscle fibers composed of elongated multinucleated cells supporting connective tissue and a peripheral nerve (PN) fiber. 40X

histology was as much an art as it was a science.^{17,18} Initially, GMA-embedded bone marrow cores were done in parallel with the decalcified sample embedded in paraffin. We succeeded in achieving sufficient protocol modifications to allow for next-day turnaround times for these samples in plastic.¹⁹ Bone marrow specimens for both the paraffin and GMA procedures were fixed in a modified B-5 fixative in which 10% neutral buffered formalin (NBF) was substituted for the more commonly used formaldehyde.²⁰ Following 2 hours in fixative, the sample intended for paraffin was decalcified in 5% formic acid. Bone marrow cores intended for paraffin were routinely decalcified overnight in 5% formic acid with a confirmed end point demonstrated with the ammonium oxalate test. This was an adaptation of procedures standardized for orthopedic specimens.²¹

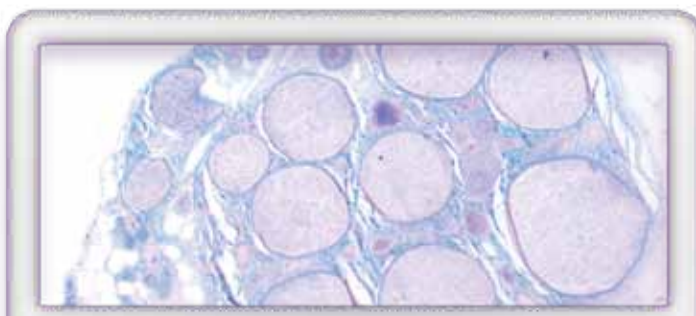


Fig. 4. A 2-micron GMA-embedded cross-section of nerve. LFB-PAS stain shows a peripheral nerve in transverse section with several fascicles, each of which contains many nerve fibers. Each fascicle is surrounded by the collagenous tissue layer (perineurium), and the entire nerve is surrounded by the epineurium. 40X

The GMA sample was dehydrated through graded ethanols to absolute alcohol before infiltration with multiple changes of catalyzed GMA monomer. With experience, we were able to reduce incubation times for all procedures in an effort to decrease turnaround time without compromising quality. However, this approach meant a potential 2-day turnaround time. Further tweaking enabled us to shorten this to next-day turnaround time. Paraffin sectioning was performed at 3 microns (Figs. 15 and 16).

The work with iliac crest wedge biopsies and cultured chondrocytes in GMA led to the development of a reliable protocol for infiltration and polymerization over a nominal 2-day

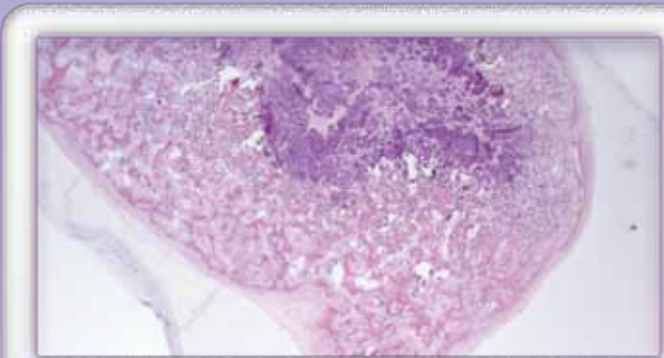


Fig. 5. A 2-micron formalin-fixed, GMA-embedded section of matrix formation in cultured rabbit growth plate chondrocytes stained with H&E (Gill's III for 10 min). This specimen type can be fixed and processed in 1.6 mL polypropylene microcentrifuge tubes to reduce manipulative distortion and maintain architecture. The matrix is growing outward from the central dark bluish purple-stained area. 20X

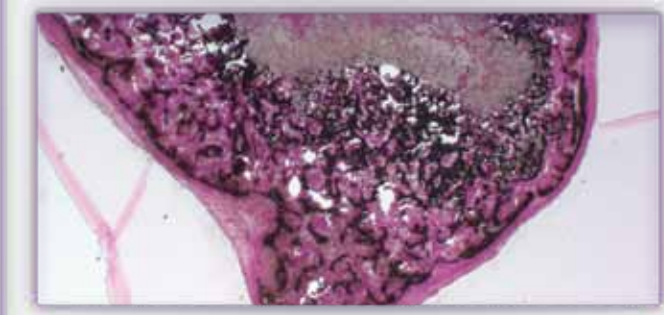


Fig. 6. A 2-micron formalin-fixed, GMA-embedded serial section of Fig. 5 stained with von Kossa. Mineralization of the bone matrix is depicted by the black staining imparted by the silver nitrate. 20X

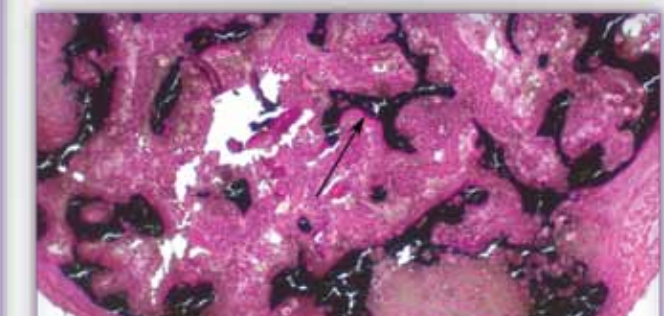


Fig. 7. Higher magnification of Fig. 6 demonstrates both mineralization (black) and nonmineralized osteoid (fuschia-red) as imparted by the acid fuchsin (arrow). Because this is newly mineralized bone, the GMA affords very intact structure to the matrix. 100X

time span. Following fixation, the specimen was dehydrated through graded ethanols to absolute alcohol for a minimum of 4.5 hours. The initial infiltration protocol for the GMA sample was to infiltrate in 2 changes of catalyzed GMA monomer with one change for 3 hours and the second change incubated overnight. We would tweak these time frames depending on the time the specimen arrived in the lab. The first change utilized a "used" monomer solution in order to get the maximum usage from the components. It was not imperative that the second change be freshly made but it had to be ethanol free. There were usually fewer than 10 samples, so a 200 mL sealed jar held enough volume for the final change.

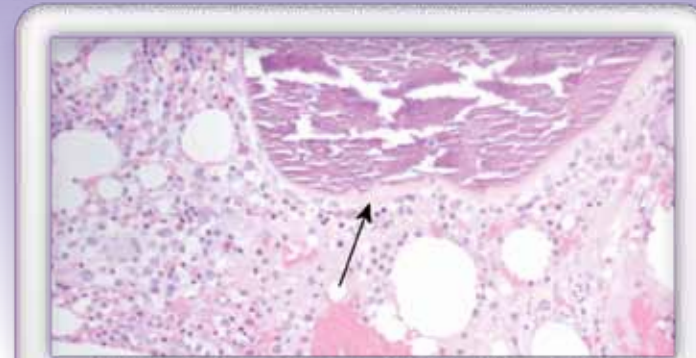


Fig. 8. A 2-micron GMA-embedded section of human iliac crest wedge biopsy. Specimen was ethanol fixed to observe tetracycline labeling and stained with H&E (Gill's III for 10 min). This section illustrates intact cellular marrow space with fat and trilineage hematopoiesis and crisp staining of the hematopoietic elements. The bony trabecula appears fractured and lifted with a zone of what looks to be osteoid at the edge (arrow). This fracturing is typical of glass knife sectioning of GMA-embedded mature mineralized bone. Using a tungsten carbide knife would improve the aesthetics. 200X

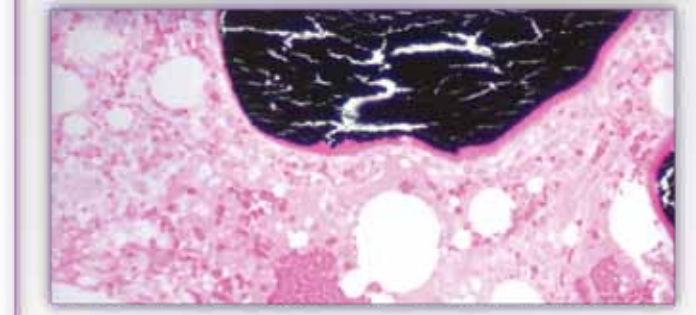


Fig. 9. A 2-micron GMA-embedded serial section of Fig. 8 stained with von Kossa, which stains the mineralized trabeculae black and clearly highlights the osteoid as fuschia-red despite the sectioning artifact. Methyl methacrylate embedment would have produced a harder block and more intact trabeculae but this did not interfere with the interpretation of the osteoid presence. 200X

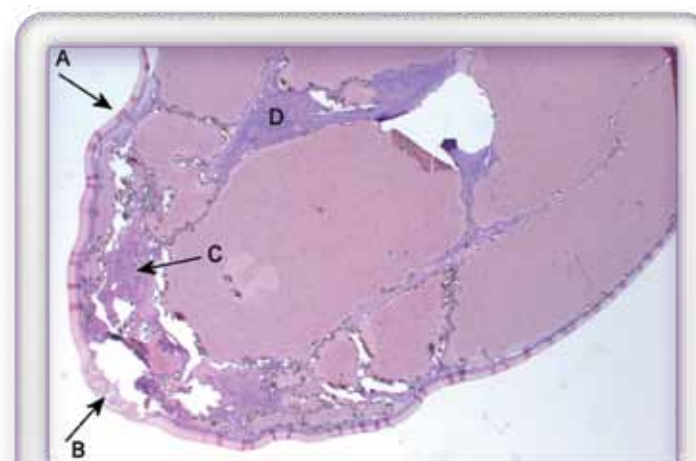


Fig. 10. A 4-micron GMA-embedded longitudinal section of a tick (*Ixodes dammini*) stained with H&E (Gill's III for 10 min). Intact ticks were fixed in formalin prior to processing. Upon sectioning, residual oil in the insect's shell would cause the body to fall out of the GMA frame, necessitating direct manipulation of the specimen, making it difficult to achieve a totally intact section. Up to 18 of these frameless sections could be placed on each slide to ensure capturing the area of interest. This section shows (A) a somewhat "crinkled" exoskeleton, (B) mouth parts, (C) salivary glands, and (D) the mid gut and red blood cell collections with hemosiderin deposits. Giemsa and Dieterle silver stains were also performed through the GMA resin. 2X

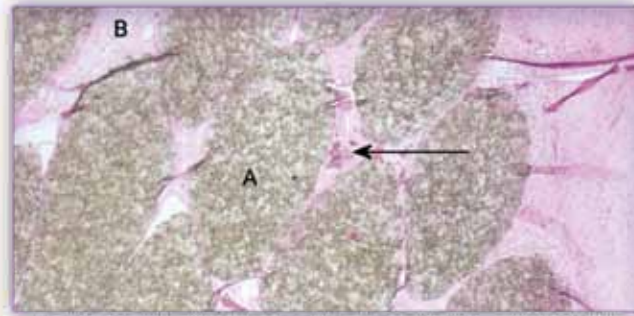


Fig. 11. A 2-micron GMA-embedded cross-section of expanded polytetrafluoroethylene biomaterial in bone that was formalin fixed prior to processing and stained with H&E (Gill's II for 15 min). The grey refractile biomaterial (A) implanted more than 4 years earlier shows an ingrowth of a cellular matrix containing connective tissue (B) surrounding the fiber filaments and, in some instances, into the filaments themselves. A potential bone island is noted (arrow). 40X

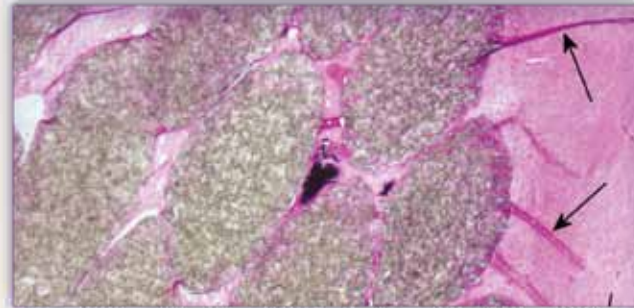


Fig. 12. A 2-micron GMA-embedded serial section of Fig. 11. Stained with von Kossa, bone fragments and areas of mineralization adjacent to graft material are highlighted by black staining. Note the wrinkles (arrows), which are an indicator of the resin being "too soft" to accommodate the various densities of the biomaterial/soft tissue construct. 40X

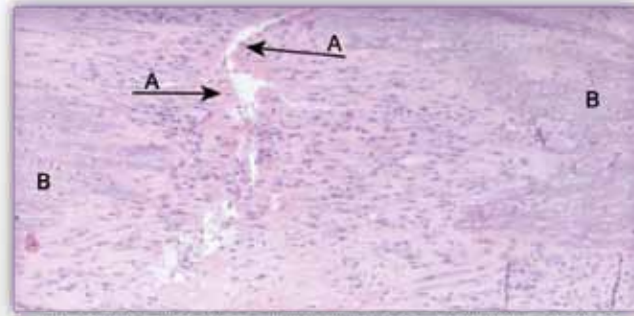


Fig. 13. A 2-micron formalin-fixed, GMA-embedded section of undecalcified regenerative bone through a distraction osteogenesis gap in rat stained with H&E (Gill's II for 15 min). Areas most proximal to either side of the midline of the fibrous interzone contain the least mature regenerating bone cells (A), while the more mature, potentially more mineralized tissues are farther away (B). 2X

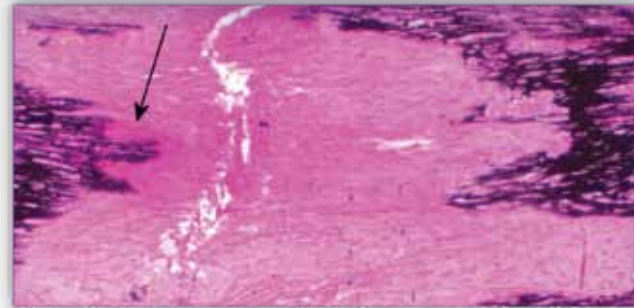


Fig. 14. A 2-micron formalin-fixed, GMA-embedded serial section of Fig. 13 stained with von Kossa. Mineralized fronts are denoted by the black silver staining. Note the higher presence of osteoid (fuscia-red staining) on one side (arrow). 2X

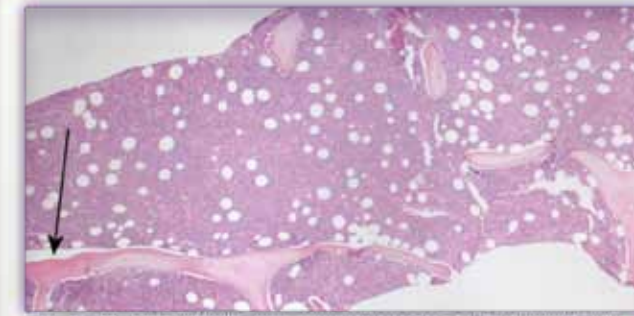


Fig. 15. A 3-micron B-5 variant fixed, formic acid decalcified, paraffin-embedded section of human iliac crest from a 2-mm diameter bone marrow biopsy. Section was stained with H&E (Gill's II for 3 min). Section shows bony trabeculae with intact cellular marrow spaces containing hematopoietic elements and fat. Removal of the specimen from the 5% formic acid decalcifier before optimal end point contributed to trabecular lift (arrow), which does not compromise diagnostic value. 40X

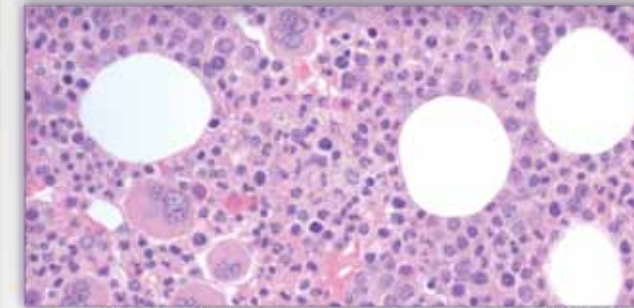


Fig. 16. Higher magnification of Fig. 15. The H&E-stained section shows nuclear and cytoplasmic detail allowing for adequate morphologic evaluation of megakaryocytes (lower left), and admixed erythroid and myeloid progenitors. 400X

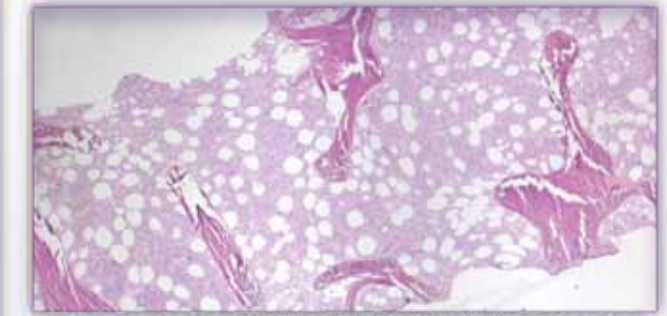


Fig. 17. A 2-micron B-5 fixed, undecalcified, GMA-embedded section of human iliac crest from a 2-mm diameter bone marrow biopsy. Section was stained with H&E (Gill's III for 10 min). Section shows intact bone marrow space and fractured bone sectioning artifact common to GMA-embedded samples of mature bone sectioned with a glass knife. 40X

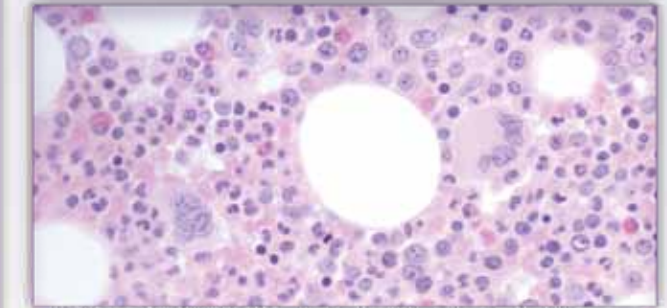


Fig. 18. Higher magnification of Fig. 17. Thin sections with enhanced nuclear and cellular features allow for differentiation of different myeloid progenitor cells (myelocyte, metamyelocyte, and band and segmented forms) and distinction of early myeloid from erythroid precursors. Eosinophils with refractile cytoplasmic granules are easily identified. 400X

Embedding of bone marrow specimens was always at the aforementioned 40:1 ratio. Blocks were sectioned at 1.5 to 2 microns using the Sorvall JB-4 triangular glass knife system (Figs. 17 and 18).

Our diagnostic criteria for bone marrow biopsies required a core that was 1 cm long at a standard diameter of 2 mm with at least 4 areas of intact marrow space. The ability to clearly identify cellular lineage and maturation pattern and a fat-to-cellular marrow ratio is considered essential. The pathologist must be afforded the clearest cellular detail possible to provide the most accurate diagnosis. Each of the depicted specimens has flaws in its presentation, most notably in the trabecular bone. The paraffin section shows less than optimal decalcification and some lift in the trabecular bone (Fig. 15). The GMA section displays shattering of the trabecular bone throughout (Fig. 17). Neither of these features interferes with the interpretation of the cellular marrow. The high-power photomicrograph of the paraffin section (Fig. 16) illustrates acceptable cellular detail, however, the GMA prep (Fig. 18) affords the observer a superior level of nuclear and cytoplasmic clarity. So, while the GMA technique is absolutely advantageous for hematopoietic studies, it has potential drawbacks for orthopedic and endocrine histomorphology due to both diminished architectural integrity at the trabecular bone-cellular interface and to interference with the ability to ascertain cellular populations such as osteoblasts and osteoclasts as they directly relate to these structures.

Conclusion

This discussion illustrates how one can integrate resin technology into a paraffin-oriented laboratory. As new biomaterials appear in research and clinical laboratories, it can be helpful to have an understanding of the utility of a basic resin such as the JB-4 GMA kit to help optimize the information to be gleaned from such nonroutine samples. In some instances, a unique specimen combined with a specialized processing strategy could lead to a new routine, as was the case with bone marrow specimens in our lab. In an effort to keep it simple, we initially relied on materials and equipment that were readily available and, because our samples were fairly small in size and number, we were able to avoid the need for more specialized equipment such as water baths and nitrogen chambers for heat regulation and the deoxygenating used in polymerization processes.^{22,23}

The samples we prepared with this technique were typically no larger than 1.0 x 1.4 cm. For larger, more mineralized samples or more complex biomaterials, one should consider incorporating methyl methacrylate methods into the laboratory repertoire.

Acknowledgments

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ID Gram Stain: A Modified Brown-Hopps Method for Gram-positive and Gram-negative Bacteria

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Abstract

Conventional Gram stains on tissue sections demonstrate gram-positive organisms adequately. Gram-negative organisms, however, often appear dull and washed out. This report discusses a modification of the Brown-Hopps technique¹ that detects a wide spectrum of microorganisms in tissue sections. This method provides equal staining intensity in both gram-positive and gram-negative microbes, reducing the possibility that a gram-negative infection might go undetected.

Introduction

Since the time when Hans Christian Gram first developed the technique for heat-treated smears that we today refer to as the Gram stain,² numerous modifications have appeared in the scientific literature for applying the technique to tissue sections. The original four-step technique reported by Gram works very well on smears of exudate or imprint smears of tissue (Figs. 1, 2); however, it is unsuitable for bacterial staining in tissue sections because connective tissue and other tissue elements readily stain with the dyes used in the technique, making any bacteria that may be present in the tissue section difficult to detect with a microscope.³ This sets the stage for a bacterial infection to go undetected.

This modified method incorporates the use of crystal violet,⁴ a cationic blue dye for staining gram-positive organisms, which is retained in the thicker cell wall of these organisms following mordanting with an iodine solution. Any number of cationic or anionic red dyes have been substituted in the staining of gram-negative organisms, such as basic fuchsin,⁴ acid fuchsin, safranin O, and neutral red. Some techniques incorporate

a third dye, such as fast green (Gram-Twort), tartrazine, or metanil yellow, to provide better contrast between the microbes and the background.³

Virtually all Gram stain variants are tricky to perform and require a standardized protocol in the hands of an experienced technologist as there are many opportunities for the staining outcome to be less than optimum.⁵ A common complaint is that gram-negative organisms may appear washed out or too lightly stained, setting the stage for the microbes to be missed when viewed with a microscope.

Materials and Methods

The technique reported here represents an attempt to shorten and simplify the staining process while intensifying the contrast between the blue and red dyes.

Modifications to the original Gram stain technique

1. Staining times were shortened from 5 minutes to 2 minutes. Crystal violet and basic fuchsin are quickly and readily taken up by the microbes.
2. Dilute Bobon's Ziehl-Neelsen (BZN) stain was used instead of aqueous basic fuchsin. This highly dilute stain provides greater specificity and less background staining. The phenol in this solution seems to "push" the stain into microbial cell walls. Gallego differentiating solution fixes the red dye (BZN) in the gram-negative cell wall, reducing the bleeding of the dye during subsequent dehydrating alcohol rinses.⁶
3. Finally, metanil yellow was used in place of tartrazine to achieve a lighter yellow counterstain.

Control: Formalin-fixed, paraffin-embedded tissue, known to be positive for gram-positive and gram-negative bacteria

Fixation: 10% neutral buffered formalin (NBF)

Section thickness: Optimal section thickness is 4 to 6 microns

Solutions:

1. 1% Aqueous crystal violet

Crystal violet dye powder	1.0 g
Deionized water	100.0 mL

2. Gram's iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Deionized water	300.0 mL

Dissolve the iodine and potassium iodide in a small aliquot of water and bring the total volume to 300 mL.

3. Cellosolve

4. Ziehl-Neelsen carbol fuchsin

Basic fuchsin	1.0 g
Absolute alcohol	10.0 mL
5% phenol, aqueous	100.0 mL

Mix well and filter before use.

5. Bobon's Ziehl-Neelsen (BZN) stain

Ziehl-Neelsen carbol fuchsin	2.0 mL
Deionized water	50 mL

Dilute 2.0 mL of Ziehl-Neelsen carbol fuchsin with 50 mL of deionized water.

6. Gallego differentiating solution

Deionized water	50.0 mL
Concentrated formaldehyde (37%-40%)	1.0 mL
Glacial acetic acid	0.5 mL

7. 0.25% Aqueous metanil yellow solution

Metanil yellow dye powder	0.25 g
Deionized water	100.0 mL

Procedure: All slides were stained on a horizontal staining platform to prevent cross-contamination between slides

All solutions from Poly Scientific R&D Corp (Bayshore, NY).

STEP	PROCEDURE	TIME
1	Deparaffinize and hydrate sections to deionized water	
2	Flood slides with 1% aqueous crystal violet solution	2 minutes
3	Rinse in deionized water	
4	Flood slides with Gram's iodine	2 minutes
5	Rinse in deionized water	
6	Differentiate in Cellosolve until blue in section disappears	
7	Rinse in deionized water	
8	Flood slides with BZN stain	2 minutes
9	Rinse in deionized water	
10	Flood slides with Gallego differentiating solution	2 minutes
11	Rinse in deionized water	
12	Flood slides with 0.25% metanil yellow solution	1 minute
13	Rinse in deionized water	
14	Dehydrate, clear, and mount	

Results:

Gram-positive bacteria—deep blue
Gram-negative bacteria—red
Nuclei and yeast spores—light red
Elastic fibers—purple
Background—yellow

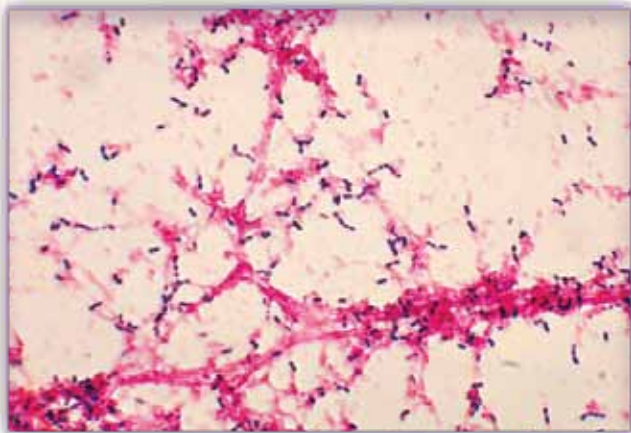


Fig. 1. Blood culture specimen demonstrating gram-positive cocci from *Streptococcus pneumoniae*. Gram stain, 1000X. Image courtesy of Dr. Mike Miller, Centers for Disease Control and Prevention Public Health Image Library. Image #2896. http://phil.cdc.gov/phil_images/20030114/13/PHIL_2896_lores.jpg.

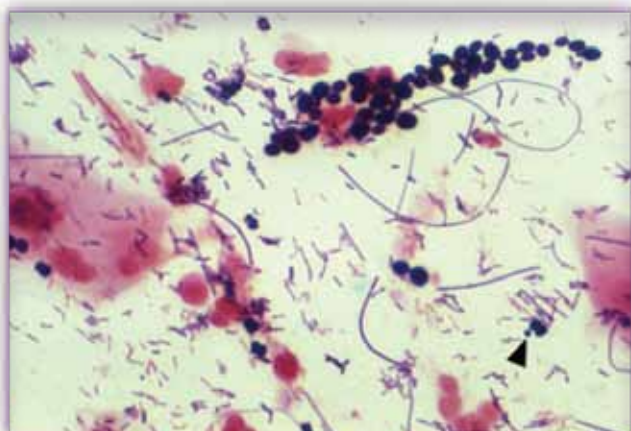


Fig. 2. Vaginal discharge specimen illustrating gram-negative rods and gram-positive budding yeast forms of *Candida albicans*. Gram stain, 1000X. Image courtesy of Joe Miller, Centers for Disease Control and Prevention Public Health Image Library. Image #15641. http://phil.cdc.gov/PHIL_images/15641/15641_lores.jpg.

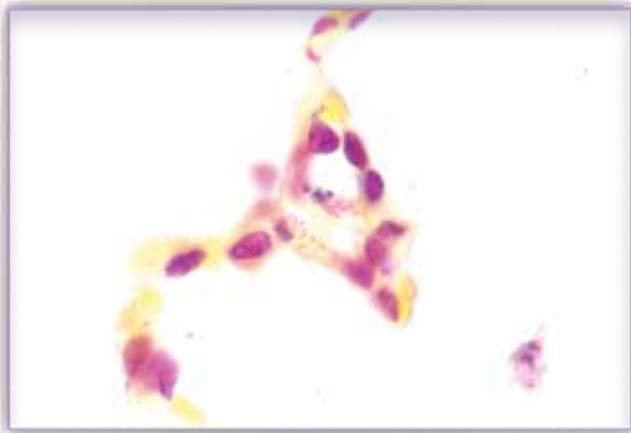


Fig. 3. Author's ID modified Brown-Hopps Gram stain of lung tissue provides a clean background, offering ideal contrast for demonstrating bacteria. 1000X

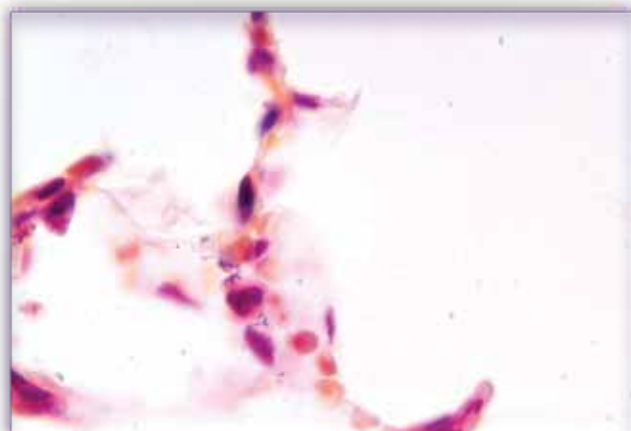


Fig. 4. Lung tissue stained with traditional Brown-Hopps Gram stain. Gram-negative organisms are difficult to see against the pink background in this slide compared to the clean background in Fig. 3, which was stained with the modified Brown-Hopps Gram stain. 1000X

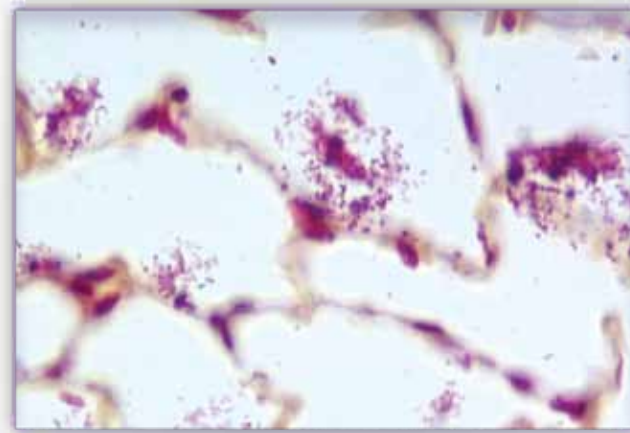


Fig. 5. Brown-Brenn commercial stain kit performed in lung control tissue, illustrating the difficulty in achieving intense staining of gram-negative bacteria. 600X

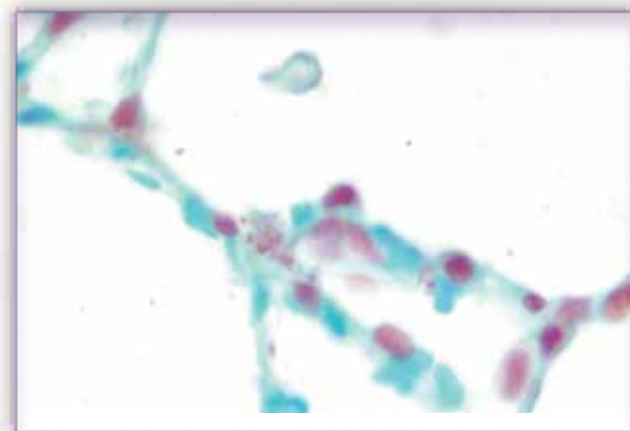


Fig. 6. Gram-Twort stain of lung tissue performed using a staining instrument illustrates the difficulty in distinguishing gram-negative bacteria from background. 1000X

Discussion

Pathologists often use the tissue Gram stain as a simple screening tool for the presence of bacteria rather than as an attempt to truly characterize and classify the organisms. Interpretation of the tissue Gram stain presents a unique set of problems, so caution must be used. Bacteria found in areas of extensive necrosis may be dead or dying, causing damage to cell walls; as a result, their staining will be variable. Bacteria that retain the crystal violet-iodine complex can safely be characterized as gram-positive. However, it is often impossible to tell whether red-stained organisms are truly gram-negative or represent dead or dying gram-positive bacteria.⁵

Gram stain variants such as the Brown-Hopps, Brown-Brenn, and Gram-Twort techniques have been reported in the literature. These methods can be challenging in inexperienced hands, leading to inconsistent results and washed out organisms that may go undetected. This problem is compounded by the fact that tissue uptake of the dyes used in these methods can create a background appearance that may mask the presence of bacteria, as demonstrated in Figures 4, 5, and 6.

The method reported here has been consistently reliable in our laboratory and offers a shorter time investment for achieving acceptable staining results (Fig. 3). It is advisable in all modifications of the Gram staining technique to dehydrate the stained sections very quickly without having them linger in alcohols in order to avoid unintentionally pulling out the red dye.

Conclusion

The modifications to the Brown-Hopps method recommended in this report provide crisp, clear, and precise differentiation of gram-positive and gram-negative microorganisms. The tissue sections stained with this method can be interpreted with confidence. We have also found that the phenol in the BZN counterstain solution extends its shelf life and prevents microbial growth in the prepared reagents, which also helps to ensure reliability with this procedure.

Acknowledgments

Mr. Larry Bobon (Bo) was my supervisor and mentor for 23 years. I am indebted to him for his professional influence, which contributed to my growth in the histology discipline. I am also indebted to Gus Mondragon, HT(ASCP), for editing this manuscript.

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Unusual Exogenous Pigmentation in Frontal Gingiva: A Brief Case Report

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Abstract

A dark, focal pigmentation was observed in the gingiva of an adult male who appeared for a routine dental assessment. Despite assurances from the patient that he had had this pigmentation since childhood, concern for the possibility of a pigmented disease process led to a biopsy that revealed the presence of an unexpected exogenous black pigment in histologic sections. This brief report discusses the etiology of gingival pigmentation and an uncommon presentation of graphite carbon in gingival tissues.

Introduction

Modern society tends to associate pink gums with healthy gums, which are most apparent during the act of smiling, an important form of communication used between individuals to convey expressions such as joy, success, sensuality, affection, self-confidence, and kindness. However, natural dark pigmentation of the gingiva, typically the result of melanin deposition in the oral mucosa, is considered quite normal and can be observed in individuals of various races throughout the world, with an apparent correlation to the degree of pigmentation in the skin.¹ Although considered cosmetically undesirable in some societies, dark pigmentation of the gingiva is usually no cause for concern; however, dental practitioners must consider the possibility of a melanocytic disease process, notably malignant melanoma, in patients presenting with dark pigmentation of the gums.

Pigmentation of the gingiva from exogenous sources (those not occurring naturally in the body) is most often attributed to the use of amalgam in various dental procedures, which can result in what is referred to as a "tattoo" of the oral mucosa (Fig. 1). In dentistry, amalgam is an alloy of mercury with various metals, commonly silver, tin, and copper, used for dental fillings.² Although reported to have been in use in the Far East since the 6th century, its popularity in recent decades has diminished somewhat out of concern that the mercury constituent in amalgam can leach into surrounding tissues. While a number of published studies demonstrate that mercury levels in various organs are higher in individuals with amalgam fillings,



Fig. 1. Gingival amalgam tattoo. Photo courtesy of Dr. Ronald B. Orr, DDS, MPH, Belmont Periodontics, PC, Belmont, MA.

there remains little scientific agreement supporting concerns that mercury exposure from amalgam dental fillings is attributable to human disease.⁴

Deliberate gingival tattooing is prevalent in some cultures of the African continent. Traditional gingival tattooing, practiced in Ethiopia and occasionally in other African and Middle Eastern nations, is performed for aesthetic appeal or superstition, and it occasionally may be used as a homeopathic remedy for dental diseases.^{5,6} In these instances, carbon sources such as soot may be used to achieve the desired result.

This report discusses an unusual presentation of black exogenous pigmentation of the frontal gingiva reportedly due to exposure to graphite carbon in childhood.

Discussion

Gingival pigmentation is not an uncommon finding. It may be of endogenous or exogenous origin and, in some instances, can have diagnostic significance.⁷

A 61-year-old white male presented at the university's dental practice with advanced periodontal disease. During the oral surgeon's presurgical assessment, he observed a black pigmented discoloration approximately 1.0 cm in diameter on the gingiva just below the front incisors of the mandible.

The patient suggested that the discoloration was the result of an amalgam tattoo, however, the surgeon noted that amalgam fillings are not used in front teeth, so the discoloration could not be the result of silver amalgam staining of the gingiva.

The surgeon suggested a biopsy despite the patient's report of having had the discoloration since childhood. The patient eventually confessed that as a child he had used the sharpened end of a graphite "lead" pencil to probe into the gingiva in that same location. Somewhat embarrassed at having to admit to this childhood act, the patient continued to insist that a biopsy was unnecessary. However, the surgeon insisted that a biopsy was necessary to rule out the possibility of an underlying melanocytic lesion (melanoma).

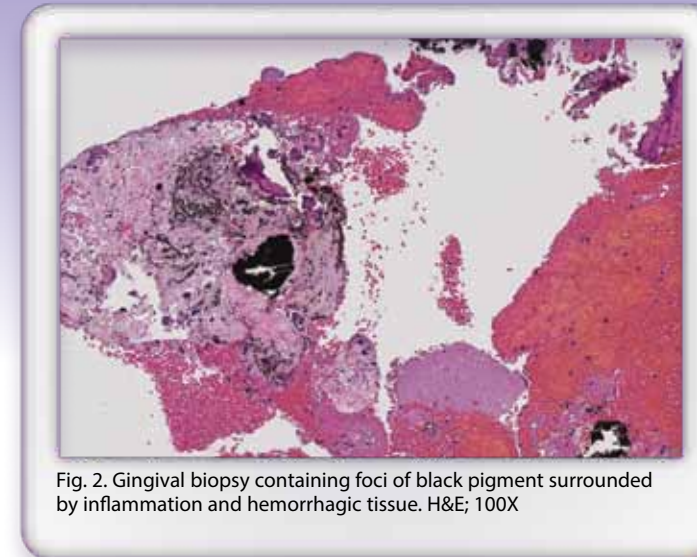


Fig. 2. Gingival biopsy containing foci of black pigment surrounded by inflammation and hemorrhagic tissue. H&E; 100X

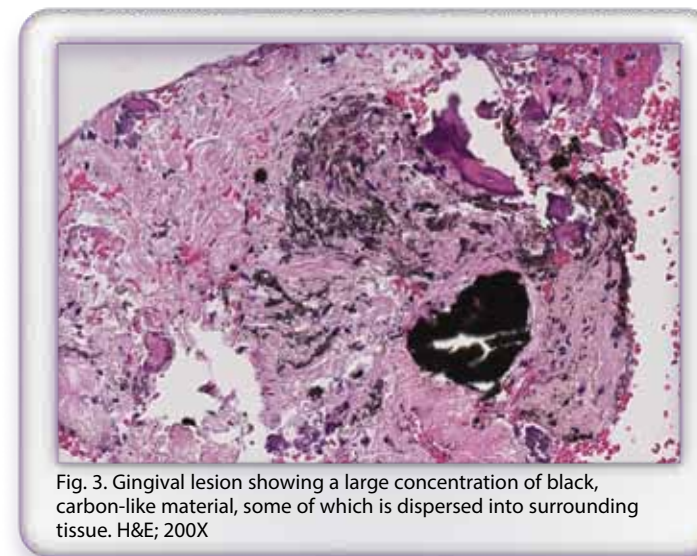


Fig. 3. Gingival lesion showing a large concentration of black, carbon-like material, some of which is dispersed into surrounding tissue. H&E; 200X

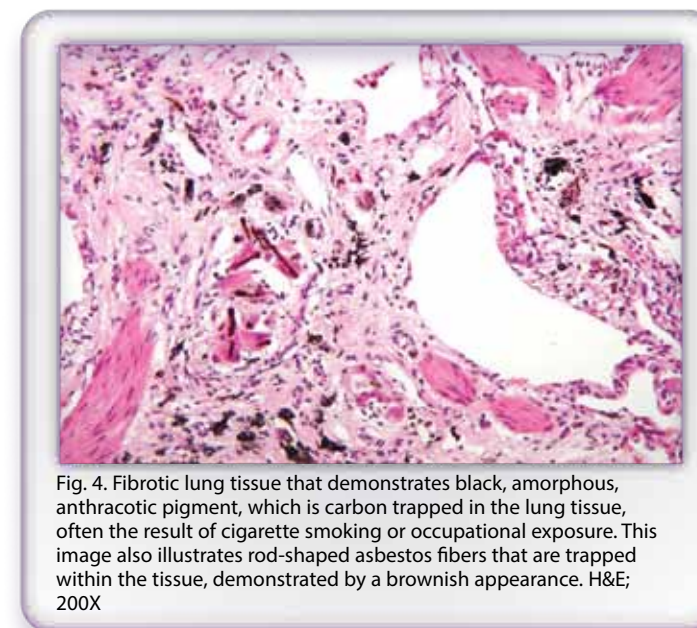


Fig. 4. Fibrotic lung tissue that demonstrates black, amorphous, anthracotic pigment, which is carbon trapped in the lung tissue, often the result of cigarette smoking or occupational exposure. This image also illustrates rod-shaped asbestos fibers that are trapped within the tissue, demonstrated by a brownish appearance. H&E; 200X

The surgeon excised a portion of gingiva and bone in multiple fragments that measured 0.7 x 0.6 x 0.2 cm in aggregate, all of which were placed into 10% formalin and sent to the oral pathology laboratory for sections. Upon examination, a dark-black pigmentation associated with apparent inflammation was observed in hematoxylin and eosin (H&E)-stained histologic sections (Figs. 2 and 3). It is unclear if the accompanying inflammation was the result of periodontal disease or the body's reaction to the exogenous pigment observed in the sections.

In histology, carbon pigment is often seen in the lungs of coal miners as anthracotic pigment (Fig. 4) and is attributed to occupational exposure, but it may also be found in the lungs of tobacco smokers and city dwellers where air quality is rather poor.⁸ Because it is extremely inert and unreactive, carbon fails to be demonstrated with conventional histological stains and histochemical methods. Carbon may be easily confused with melanin deposition; however, the distinction can be made by using melanin bleaching agents, which will not affect carbon but will dissolve melanin.⁸

"Lead" pencils do not contain the element lead at all, but rather are made using a form of carbon called graphite, a mineral found in nature that is also used for lubrication of metals. In tissues, graphite is an insoluble, nonrefractile black pigment that typically remains in the location where it is introduced. Historically, solid deposits of graphite in nature were originally thought to be lead, and this term is still used today to refer to solid graphite.

Conclusion

The observation of dark pigment in the gingiva of otherwise healthy individuals is not normally cause for concern, however, the possibility of underlying melanocytic disease must be considered. It is common for the histology laboratory to encounter endogenous and exogenous pigments in tissues under study. Although uncommon outside of the lungs, carbon deposits are insoluble and may be confused with other pigments such as melanin, which should be investigated to rule out underlying disease including melanoma.

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22 **NSH Webinar 1:00 pm Eastern Time**
 Title: **Principles of Perinatal Autopsy**
 Speaker: Jacqueline Macknis, MD
 William Beaumont Hospital
 Royal Oak, MI
 Phone: (443) 535-4060 or register online at www.nsh.org

NOVEMBER

5 **NSH Webinar 1:00 pm Eastern Time**
 Title: **The Magical Science of Rubrics**
 Speaker: Joyce Sohrabian, HT(ASCP)
 Argosy University
 Eagan, MN
 Phone: (443) 535-4060 or register online at www.nsh.org

26 **NSH Webinar 1:00 pm Eastern Time**
 Title: **What Happened to My IHC & What Caused It?**
 Speaker: Jesse DelCampo, HT(ASCP)
 Biocare Medical
 Concord, CA
 Phone: (443) 535-4060 or register online at www.nsh.org

DECEMBER

17 **NSH Webinar 1:00 pm Eastern Time**
 Title: **Human Epidermal Growth Factor Receptor 2 (HER2) Testing: Validation, Application, and Correlation**
 Speaker: Joelle Weaver, MS, HTL(ASCP)QIHC
 PGXL Laboratories
 Louisville, KY
 Phone: (443) 535-4060 or register online at www.nsh.org



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