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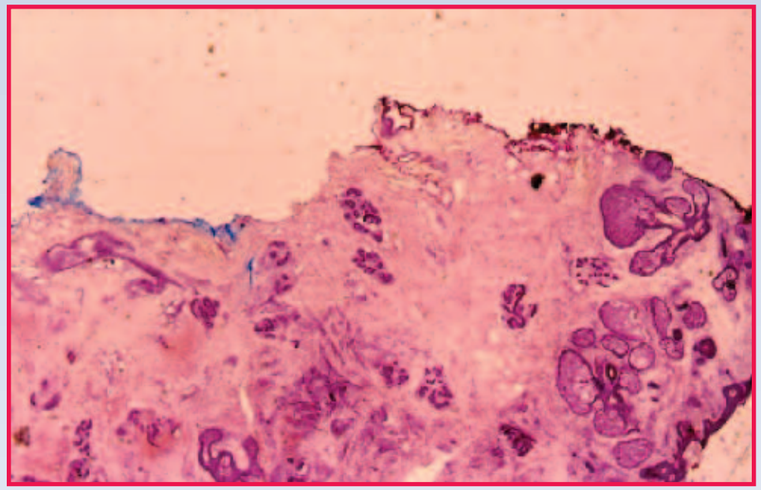

Fig. 1. 1999 H&E section pretreated for 15 minutes with 0.5% sodium hypochlorite immersion. 40X

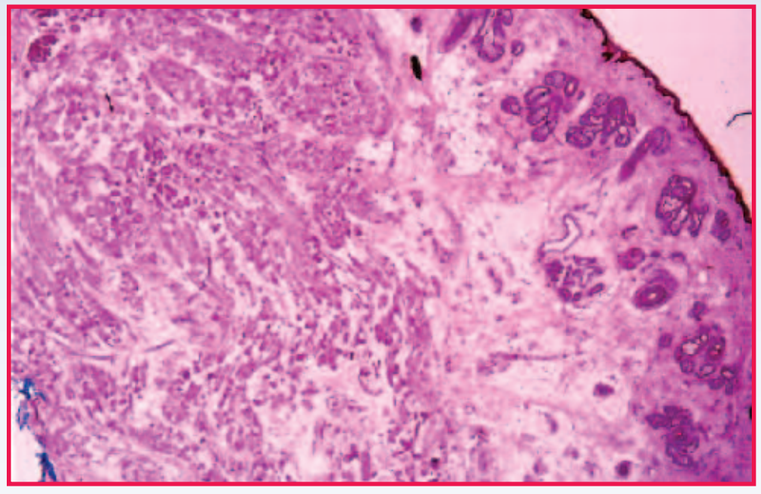

Fig. 2. 2000 H&E section pretreated for 15 minutes with 0.5% sodium hypochlorite immersion. 100X

Sodium Hypochlorite Soak for Freshly Excised Skin Tissue Prior to Grossing and Frozen Sectioning

Mechanism of Action as an Antiviral and Antimicrobial Agent and its Effect on Tissue Components and Staining

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Abstract

Histology and Mohs surgery laboratories frequently work with fresh unfixed patient samples that may pose a significant infectious risk to laboratory personnel. While fixation of tissues is thought to render them noninfectious, some

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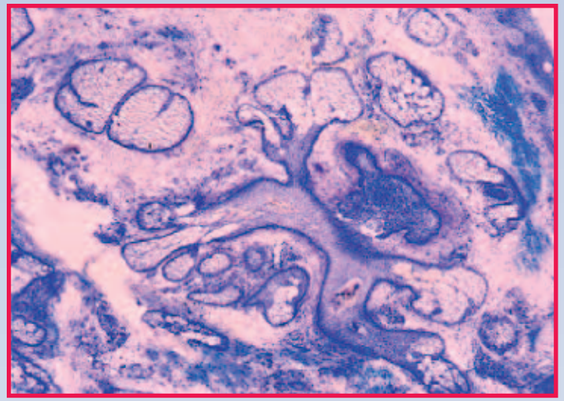

Fig. 3. 2000 Toluidine blue section pretreated for 15 minutes with 0.5% sodium hypochlorite immersion. 100X

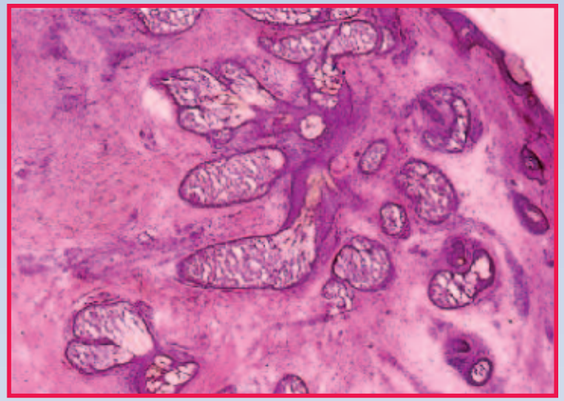

Fig. 4. 2001 H&E section pretreated for 15 minutes with 0.5% sodium hypochlorite immersion. 100X

laboratory techniques require that the samples remain unfixed for testing. While laboratory personnel understand the risks associated with working with fresh unfixed samples, there is still heightened anxiety that can be a source of distraction, possibly leading to an accidental exposure. We report a technique for adopting the prophylactic use of a 1:10 aqueous dilution of household bleach (5.25% sodium hypochlorite) as an antimicrobial and antiviral agent prior to the preparation of frozen sections, to reduce or eliminate risk to laboratory personnel. We also demonstrate the effects of sodium hypochlorite on fresh tissue for frozen sectioning from randomly selected stained slides collected over a 6-year period.

Introduction

In 1984, the CDC and NIH jointly published a set of guidelines for the safe handling of blood-borne pathogens.[1] Hepatitis B (HBV), hepatitis C (HCV), and human immunodeficiency virus (HIV) are among some of the transmissible agents that have been identified as posing a risk to laboratory personnel. While the CDC discourages the preparation of frozen sections on fresh frozen tissues from HIV patients, it recognizes that this testing may be necessary in some circumstances and has established decontamination guidelines for both the cryostat and laboratory countertops.[2] The virus can be introduced into any break in a staff member's skin during trimming of tissue for histological processing. To prevent infection in laboratory workers, standard precautions promulgated by CDC and OSHA must be observed when working with fresh tissue samples.

Sodium hypochlorite is an important reagent used in many disciplines including being universally employed as a decontaminant for work surfaces; an antimicrobial and antiviral agent in dentistry, veterinary medicine, and wound management; and as a fixative in human pathology, including tissue procurement; and marine and plant biology. Its mechanism of action will be explored.

Although little or no data are available on the prophylactic use of sodium hypochlorite as an antiviral or antimicrobial agent for fresh tissue prior to frozen sectioning and its effects on processing and staining, we believe reasonable conclusions can be made from data relating to its mechanism of action.

Sodium hypochlorite can be used in the following scenarios.

a) As a decontaminant of transmissible diseases such as HIV, sodium hypochlorite can be used on work surfaces and instruments. Instruments are decontaminated in 15 minutes; work surfaces require 30 minutes of contact with the solution.[3]

b) Sodium hypochlorite is the most widely used irrigating solution in endodontics. It is both an antimicrobial and tissue dissolving agent used in root canal procedures to dissolve pulp. Bovine pulp dissolves in 5.25% sodium hypochlorite, or household bleach, in as little as 20 minutes and up to 2 hours.[4]

c) Human brains infected with Creutzfeldt-Jakob disease (CJD) are immersed in formic acid and sodium hypochlorite solutions greater than 2%. Two to three days may be needed to deactivate prions that occupy the entire brain surface. Sodium hypochlorite can eliminate prions on work surfaces if left undisturbed for 15 minutes.[5]

d) In marine biology tests, sea cucumbers were dissolved in a test tube filled with sodium hypochlorite during a 24-hour immersion.[6]

e) Tobacco seeds, treated with 0.5% sodium hypochlorite are sterilized in 5 minutes.[7]

f) Dakin's solution (another name for 0.5% hypochlorite with a boric acid buffer) has long been used to kill germs and prevent germ growth in wounds. It is still used but is no longer recommended because it is cytotoxic and inhibits granulation.

Similarly, we have adopted a common practice of wetting or soaking fresh, known infectious specimens for frozen sectioning in a 1:10 aqueous dilution of household bleach for a short duration prior to grossing and cutting. All other specimens are soaked for 5 minutes. The known infectious samples, such as hepatitis B and C, HIV, Kaposi's sarcoma, and genital herpes and warts, are soaked for 15 minutes. When observed microscopically, no adverse effects on tissue components, frozen preparation, or staining were noted.

Materials and Methods

Standard methods of fixing small tissues (5 mm to 1 cm) facilitate penetration. We know that matrix hardening fixation for paraffin blocks requires 1 hour per millimeter of tissue thickness. Traditionally, frozen sections cut on a freezing microtome are fixed in 10% formalin for only 1 to 2 minutes before sectioning.[8] The volume of fixative should be 20 times the volume of tissue.[9]

Surgically excised Mohs tissue is received in the lab. Since the tissue can begin to float in the pan when immersed, the superior 12 o'clock surface is first marked with gentian violet to preserve orientation.

Known or suspected infectious tissue is immersed in a pan containing freshly prepared 0.5% sodium hypochlorite (one part household bleach to nine parts distilled water); it is placed under a fume hood for 15 minutes. All other specimens that come into the lab are routinely flooded with the solution for 5 minutes using a squeeze bottle. Tissue is then blotted dry, dyed, grossed, and processed by frozen section method, retrieved onto coated slides, and stained. Standard precautions are observed during processing.

Results

Randomly selected slides from 1999 to 2005, prepared from excised human skin that had been soaked in a solution of 0.5% sodium hypochlorite for 5 to 15 minutes prior to frozen section processing, were reexamined. There appeared to be comparable clarity of stain over a 6-year period for slides having been pretreated with 0.5% sodium hypochlorite. Tissue components were easily identified and H&E and toluidine blue stains retained their original hues (Figs. 1-8).

Advantages

1. 5.25% sodium hypochlorite (household bleach) is inexpensive and readily available (it also comes in a fresh scent).
2. Sodium hypochlorite suppresses, destroys, and prevents transmission of infectious agents.
3. At low concentrations, for short-term exposure, sodium hypochlorite is biologically compatible with frozen section microtomy.
4. Its suggested use as a prophylactic against infectious organisms in the laboratory may lessen fear of injury during the handling and preparation of fresh tissues.

Disadvantages

1. Sodium hypochlorite is highly corrosive and breaks down rapidly when diluted. A 1:10 dilution must be made fresh daily.
2. Sodium hypochlorite can be harmful if used by those with a hypersensitivity to chlorine bleach.
3. Tissue will dissolve completely in the solution if left for long periods.

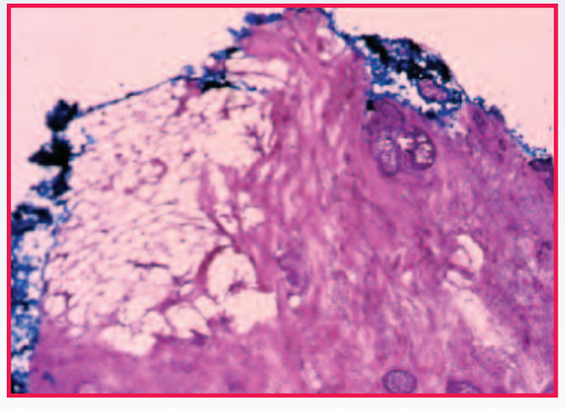

Fig. 5. 2002 H&E section pretreated for 15 minutes with 0.5% sodium hypochlorite immersion. 40X

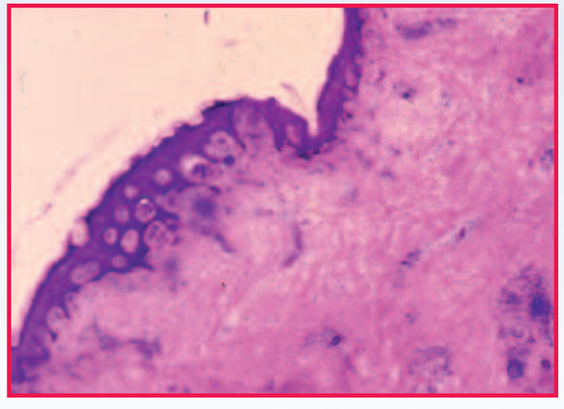

Fig. 6. 2003 H&E section pretreated for 15 minutes with 0.5% sodium hypochlorite immersion. 40X

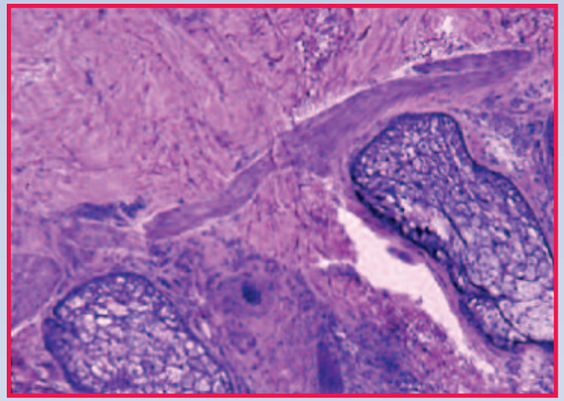

Fig. 7. 2004 H&E section pretreated for 15 minutes with 0.5% sodium hypochlorite immersion. 100X

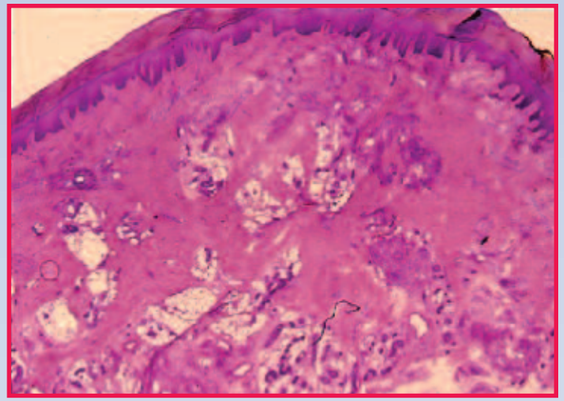

Fig. 8. 2005 H&E section pretreated for 5 minutes with 0.5% sodium hypochlorite wet flush. 40X

4. Exact "fixation" time and solution concentration needed to completely destroy infectious agents in fresh tissue excised during the Mohs frozen section method are not known and must be determined empirically.

Discussion

Discussions about the biological effects of fixatives began as early as 400 B.C. with Hippocrates. A systematic study of fixatives began only after the invention of the microscope in the late 19th century. Generally, routine fixatives preserve cells, harden the matrix, and inactivate transmissible diseases. Although 0.5% sodium hypochlorite is not a standard tissue fixative, in some cases it is noted as a special oxidizing fixative when combined with formaldehyde.[5]

In addition, it has been reported that full-strength Clorox®* Bleach added as an oxidant in the preparation of Harris hematoxylin intensifies the stain while effecting bacterial stasis of the solution.[10]

High concentrations of sodium hypochlorite rapidly destroy and dissolve organic tissue. It is highly corrosive and has a short shelf life when diluted. Other fixatives such as 95% ethanol will inactivate the AIDS virus but should be avoided, since they inhibit the freezing point of tissue. Transmissible organisms are inactivated by 10% formalin, but it leaves a black precipitate in the stained sections. Unfixed proteins are sticky and aid in bonding tissue to a slide. Formalin fixation inhibits bonding. Sodium hypochlorite (NaOCl) acts as a solvent on organic tissue. Chlorine presents antimicrobial and antiviral action by inhibiting bacterial enzymes. This can be attributed to the high pH (12), which causes chemical injuries and degradation to organic compounds.

The amino acid reaction of chloramines interferes with cellular metabolism. Oxidation speeds up irreversible bacterial enzyme inhibition.

Saponification verifies that exposure to sodium hypochlorite can cause dissolution of organic tissue. If you were to get concentrated bleach on your skin, you would feel the results of a chemical reaction—a slippery texture coming from the bleach that is degrading fatty acids in the skin—causing the formation of soap and glycerin, if the reaction continues long enough. The dissolution of tissue is directly proportional to the concentration of sodium hypochlorite and duration of exposure.

When bleach is added to water, it forms sodium hydroxide (NaOH) and hypochlorous acid (HOCl).[11] This less alkaline reaction increases microbicide activity with less tissue degradation.

In one Brazilian study, exposure tests were conducted to determine the minimum antimicrobial inhibitory effects of concentrations of solutions ranging from 0.5% to 5% with direct exposure at 5, 10, 15, 20, and 30 minutes. Organisms tested were: *Staphylococcus aureus, Escherichia faecalis, Pseudomonas aeruginosa, Bacillus subtilis,* and *Candida albicans.* The minimum concentration of sodium hypochlorite required to inactivate all microorganisms was 0.5% at all time periods.[4]

In another Brazilian study, 0.5% sodium hypochlorite foot baths were found to be effective as an antisepsis for preventing pedal infection in cattle. In addition, the treated cattle suffered no interdigital irritation or local vascular congestion from the treatment while cattle treated for an average of 20 minutes with a 1% solution did present irritation at all time intervals.[12]

Conclusion

Sodium hypochlorite rapidly dissolves tissue and destroys transmissible microorganisms. Its effectiveness as a germicidal decontaminant on porous work surfaces is documented. We have no reservations adopting it as a soaking agent for Mohs fresh tissue prior to grossing and frozen sectioning, since tissue will be discarded after microscopic evaluation.

Generally, our samples are 3 mm thick. Based on the mechanism of action in regard to tissue dissolution capacity, we believe the minimal required concentration of sodium hypochlorite for use as an antimicrobial and antiviral agent can be effective in helping prevent the spread of infectious disease in the event of injury to laboratory personnel during processing of frozen sections. Short-term fixation in 0.5% sodium hypochlorite is biologically compatible with the process and presents low toxicity to host cells. The integrity of the stain is preserved and remains vivid after 5 years. The result is a permanent slide with no fading. We find that by lowering the pH through dilution (1:10), the working solution becomes less alkaline, resulting in a vivid, long-lasting stain while preserving the integrity of the tissue.[13]

Based upon these observations, we believe that as a fixative for frozen sectioning, sodium hypochlorite offers optimal penetration of small tissues through immersion at 5 to 15 minutes in a low but adequate 0.5% dilution with water (1:10). Under these conditions, its biological compatibility presents low toxicity to host cells.

In our experience of using sodium hypochlorite to wash fresh skin samples, we find no deleterious effect on staining or significant alteration of microscopic appearance in these tissues, which suggests that the proposed sodium hypochlorite wash offers a viable strategy for reducing infectivity in fresh tissues.

Acknowledgments

We would like to thank our Mohs surgeon, Kishwer S. Nehal, MD. We would also like to thank Adriana Ward, HT(ASCP) and Yogesh G. Patel, MS.

*Clorox is a registered trademark of The Clorox Company.

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Laser Microdissection for the Histology Technician

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The practice of isolating and analyzing a single cell or cell type from stained tissue on a slide has come into mainstream use through the technology known as laser microdissection (LM), which provides molecular information about DNA, RNA, and proteins in targeted tissue samples.[1-4] Histology technicians (HTs) have been incorporated into the LM process because of their expertise in tissue handling.[5] This technique offers numerous opportunities for the optimization of handling samples. In the LM laboratory of the National Institute of Environmental Health Sciences (NIEHS), we have optimized a methodology based upon experience and needs of researchers. Frozen sections are briefly stained to identify and then remove targeted cells, which are digested in buffer. The cellular material is then enriched and analyzed for molecular information. Because morphological preservation of tissue can be greatly compromised during processing due to the speed at which the samples must be handled to protect the molecular targets of interest, we have optimized this part of the protocol. The NIEHS guidelines were configured to facilitate optimal specimen handling for pathologists and molecular biologists, in order to achieve the correct diagnosis.[6]

Protocols for LM

Protocols for LM originated from guidelines for enzymological techniques.[7] A comprehensive reference book has been published about LM, detailing molecular protocols and applications.[7] Alcohol-based fixatives increase nucleic acid yield. Digestive enzymes (or

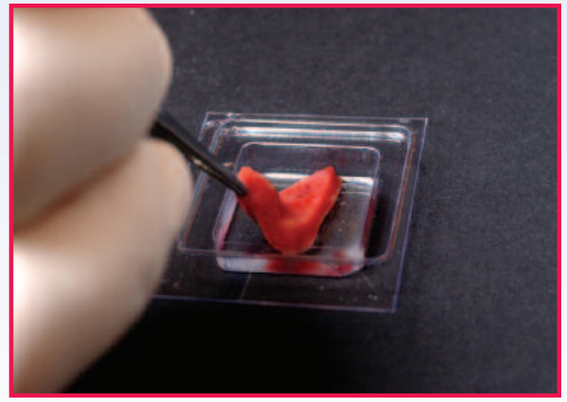


Fig. 1. In a sterile disposable cryomold, rodent liver is oriented in a small amount of Tissue-Tek® OCT.

proteases), including DNase and RNase, loosen linked cellular components. Two enzymatic manipulations include stoppage of all enzymatic activity prior to LM, and digestion of the target sample after LM. Total desiccation of the tissue section, which temporarily turns off enzymatic degradation, constitutes an ephemeral action that is most hazardous for RNA; for this reason, a maximum microdissection time of 20 minutes is recommended. While in a desiccated state, DNA is stable for much longer times.

Base solutions for all LM staining protocols start with nuclease-free water, absolute ethanol, and xylene. Fixation and dehydration steps are completed in seconds, not minutes. Because organic stains may inhibit the molecular yield, optimization is essential. Publications have documented the adverse effect that routine hematoxylin can have on molecular analysis.[8,9] Tissue sections for LM are not coverslipped, however, one of the serial sections can be used as a map slide by staining with routine hematoxylin and eosin (H&E) and then coverslipping, to allow for improved cellular identification. With these points in mind, one can see the need for preliminary testing and optimization of the protocol for each LM project.

The NIEHS LM lab starts every project by outlining expectations with the primary investigator. If the study utilizes patient samples, all Internal Review Board (IRB) consents must be in place. A clinical pathologist works closely with the primary investigator and HT to make a diagnosis, providing documentation for each sample. The HT should be prepared to discuss optimized tissue handling approaches with the surgical staff or necropsy technician. By beginning with proper sample handling procedures in the surgical suite, the process will be off to a good start.

Optimal Collection of Tissues

At NIEHS, we have a procedure that yields the best quality and greatest quantity of RNA for amplification and microarray analysis of human and rodent samples. It requires that the target sample must be handled under RNase-free conditions in a near-sterile environment. This is achieved by prepping the tissue collection area with an RNase-destroying solution (available from many manufacturers), rinsing the area well with nuclease-free water, and allowing it to dry. Sterile disposable weigh boats and sterile gauze, blades, and gloves that are changed often are a necessity. Time is critical when dealing with enzymatic activity. We have found that tissue collected and placed in Tissue-Tek® OCT™ (optimal cutting temperature) (Sakura, Torrance, CA) solution within 5 minutes is optimal. Disposable Tissue-Tek® Cryomolds® (Sakura, Torrance, CA) are used to circumvent any cross-contamination that may result from reusable cryomolds. The Tissue-Tek® Cryomold, with the specimen oriented in OCT, is placed on a metal disc that is precooled before use and then embedded in dry ice. This method helps with tissue orientation and enhances freezing in a quick and even manner before storage in the -80°C ultrafreezer.

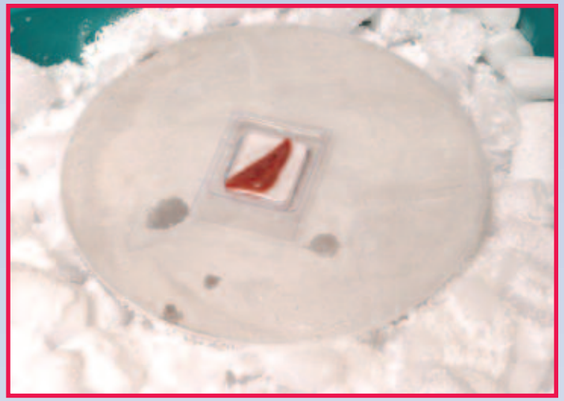


Fig. 2. Liver sample is immediately transferred onto a precooled metal disc in dry ice for rapid freezing. Proceed to cryostat sectioning for LM or transfer labeled sample to -80ºC ultrafreezer for storage.

Fig. 3. Cryostat sections are placed immediately into a slide box precooled in dry ice.

In a sterile disposable cryomold, orient tissue section with a small amount of Tissue-Tek® OCT (Fig. 1).

Immediately transfer cryomold with oriented sample onto a precooled metal disc for rapid freezing (Fig. 2). Proceed to cut frozen sections for LM or transfer sample to the -80°C ultrafreezer for storage.

To minimize and prevent desiccation at -80°C, place the labeled cryomold with tissue into a small sealed plastic bag also labeled with the sample identification. Samples for LM should not be removed from the freezer multiple times for sectioning; we have found that two removals are the maximum number before RNA integrity is compromised.

Knowing how fixation affects the molecular components in tissue is helpful in determining tissue handling procedures.[10] Enhancement of molecular kits and their protocols have enabled researchers to isolate RNA from 10% neutral buffered formalin-fixed paraffin-embedded LM samples for quantitative polymerase chain reaction (PCR).

Cutting Sections

Presently, cryostat sections are used most often in our lab. Thicker sections often prove to be less reliable in obtaining optimal LM results. A good guideline for choosing the proper section thickness is to find the thickness that allows retention of a distinct outline of a single cell among the population of assorted cells. The NIEHS baseline LM protocol directs the user to cut 8-micron sections with no more than 2 sections per slide. Mounting more than 2 sections per slide will allow previous sections to thaw partially, thus compromising RNA integrity. Less stringent tissue handling may suffice for quantitative reverse transcriptase (QRT) PCR analysis but will not prove optimal for microarray.

Different Slides for Different Instruments

The type of slide used is directly related to the laser platform. Table 1 explains the slide types used with the instrumentation available at the NIEHS lab.

For the Leica AS Laser Microdissection (LMD) instrument (Leica Microsystems, Inc, Bannockburn, IL), the polyethylene naphthalate (PEN) foil is the type of slide we use most often. The glass behind the foil membrane maintains a rigid support for the placement of frozen sections. With an immunofluorescence (IF) protocol, autofluorescence may occur with PEN foil slides. Polyethylene terephthalate (PET) or polyethylene (POL) foil membrane is the better choice. POL foil is thinner but will not withstand acetone fixation. PEN, PET, and POL foil slides are available from laser microscope manufacturers.

For the Arcturus PixCell IIe Laser Capture Microdissection (LCM) instrument (Arcturus, Mountain View, CA), the new cytogenetic slide (Erie Scientific Company, Portsmouth, NH) has recently performed well. In the past, regular uncharged slides allowed for nonspecific transfer at ambient points of nonmelting, especially problematic for tiny biopsies. To achieve minor charge but still melt and lift by focally activating the EVA with the laser, we remove the packaging from charged slide boxes, date the box, and allow the charge to dissipate for 2 to 3 weeks. This procedure has proven effective, permitting only the cells of interest to be transferred. Arcturus offers the high sensitivity (HS) cap system to overcome nonspecific transfer; however, capture area is reduced by half, and problems with pickup may occur.

For diagnosis, the first section is mounted on a fresh charged slide, stained with H&E, and coverslipped. The next 5 serial sections are placed on the appropriate slide type (Table 1) and stored immediately in a slide box partially buried in dry ice (Fig. 3).

Cutting continues in this manner: slide #6 is placed on a charged slide for H&E with coverslipping, and serial sections #7-10 are held on dry ice for LM. Once cryostat sectioning is complete, the sample is removed from the cryostat and immediately placed with the slides at -80°C for storage. These slides should be used for LM within 1 week of sectioning. This is typical of the approach we use to begin an LM project; however, needs of the investigator may warrant protocol modification, altering the number of slides to be made.

Preparing for the LM session

Fresh lysis buffer is made the morning of the scheduled LM session. Many kits are offered that include the appropriate buffer (Table 2). The expiration date should be checked and the buffer vortexed briefly before using. The slides to be stained are removed and held buried in dry ice. To obtain an RNase-free stain setup, dishes must have been cleaned with the aforementioned commercial solution, rinsed well in nuclease-free water, and air dried. For small sets of slides, sterile plastic 50-mL conical tubes can be used for one or two slides, stained back to back; 35 mL of solution is sufficient to cover the slides. Solutions need to be changed after staining 4 slides or 2 slide batches (back to back). The tubes are discarded after 1 day's use. All dilute solutions are made with nuclease-free water. Stains are made fresh and filtered. For DNA work, we use fresh Mayer's hematoxylin solution (Sigma, Atlanta, GA) filtered between tissue/project sets, and changed each day. The standard LM staining protocol follows basic histologic principles but is carried out much faster than routine histological protocols. If left in xylene for more than 5 minutes, PEN foil slides may separate. With these limitations in mind, we follow the LM protocol on the next page.

Table 1. — Types of Slides Used With NIEHS Instruments

Instrument	Laser type	Slide platform	Slide platform	Slide platform
Leica AS/LMD	UV* laser[11] /ablation 337 nm laser	PEN foil membrane mounted on frosted slide[12]	PET foil membrane on metal frame[12] • better fluorescence	POL foil membrane on metal frame[12] • best for IF • warning: acetone will dissolve POL foil
Arcturus PixCell IIe LCM	IR† laser/EVA‡[2] spot melting 810 nm laser	New frosted-end and noncharged slides	Dissipated charge from opened charged slides	New Erie Superfrost Excell™ slide (performed well with brain sections)

* Ultraviolet.
† Infrared.
‡ Ethylene vinyl acetate.

LM Protocol

1. Fix frozen section in 70% ethanol for 1 minute.
2. Rehydrate in nuclease-free water for 15 seconds.
3. Apply fresh filtered stain for 30 seconds to 1 minute.
4. Use a bluing agent (if using Mayer's hematoxylin), such as 1X automation buffer or 0.5% lithium carbonate made fresh in nuclease-free water, for 30 seconds.
5. Rinse in nuclease-free water for 30 seconds.
6. Begin dehydration, with fresh 95% ethanol made with nuclease-free water, for 30 seconds.
7. Continue dehydration with fresh high-quality absolute ethanol for 30 seconds.
8. Complete dehydration using fresh high-quality absolute ethanol for 30 seconds.
9. Clear using fresh high-grade xylene for 1 to 5 minutes.
10. Air-dry under laboratory hood for complete desiccation, 2 to 5 minutes.

The drying step is optimal for every protocol but most critical for the Arcturus cap system. Any tissue moisture will hinder the cap material (EVA) from attaching to the desired cells, resulting in unsuccessful removal. Enzymes require water, which degrades tissue components. Complete desiccation allows the maximum amount of handling time.

Our observations have shown that both RNA yields and RNA integrity are diminished by Mayer's hematoxylin. After testing several stains, we found that cresyl violet acetate (Sigma, Atlanta, GA) yields the best morphology and good-quality RNA for downstream microarray analysis. This 0.1% stain solution is made fresh and filtered using a 10-cc syringe fitted with a 0.22-micron filter unit. This protocol gives superior nuclear detail in 8-micron frozen sections of rodent brain and liver.

While waiting for desiccation, the HT can prepare the LM instrument, pipettor, barrier tips, and appropriate lysis buffer solution. Sterile 500-mL microcentrifuge tubes are used for sample collection in most studies. The details of instrument preparation can be found at the Web sites of the individual instrument suppliers.[13,14]

LM Harvesting of Cells and Image Acquisition

After LM, digestion of the harvested material by enzymatic action is performed. Table 2 provides a general guideline of buffer types used in our lab for the specific applications. Each molecular method requires a different lysing buffer. However, research may soon make it possible to have one buffer that can be used in all of the molecular methods.

Once the sample is in the proper lysing buffer, it is heated in order to activate the enzyme. The cellular components are now available for isolation and molecular analysis. Different molecular assays require different amounts of microdissected material. The molecular biologist must think in terms of picograms and nanograms for analysis and acquisition of data. Analysis for loss of heterozygosity, with DNA material, can be completed with as few as 1000 microdissected cells by PCR.[15] QRT-PCR can be run with 500 microdissected cells initially handled for RNA,[16] and a Western blot analysis can be validated from an area of microdissected tissue as small as 10^6 microns squared.

Almost all LCM publications describe the microdissection target in terms of number of cells. Often the cell count data actually represent cell fragments. A frozen section cut at 8-10 microns may not contain whole cells. The total number is an approximation at best (see Table 3).

The final element to consider is image acquisition. LM instruments offer imaging software to enhance accurately the molecular data. Most publications show the map (H&E, coverslipped) slide with a designated

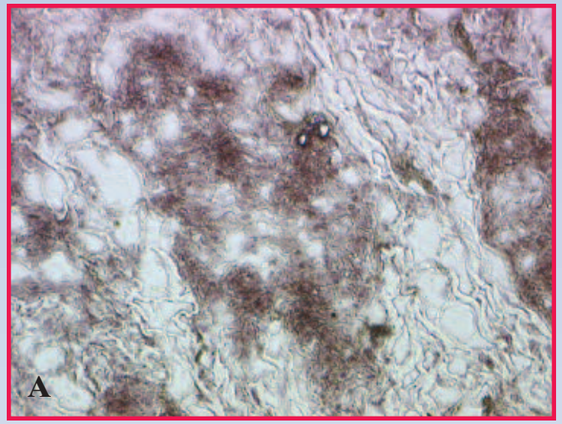
Table 2. — Types of Buffers for Specific Analysis

Microdissected material to be analyzed	Lysing buffer
DNA	Proteinase K-containing buffer
RNA	β-mercaptoethanol-containing buffer
Protein	IEF* for protein identification or SDS† buffer for Western blot analysis

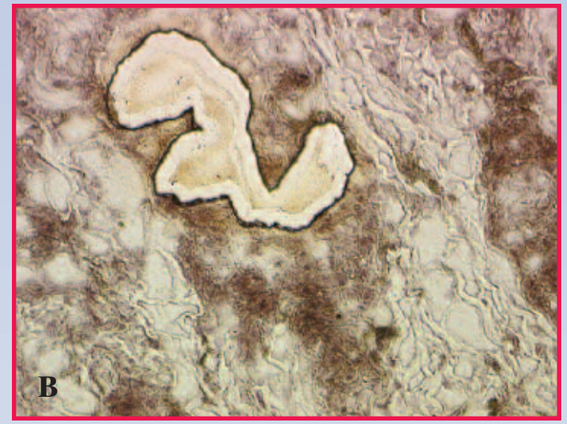
* Isoelectric focusing.
† Sodium dodecyl sulfate.

Table 3. — Microdissected Area Given by NIEHS Instruments

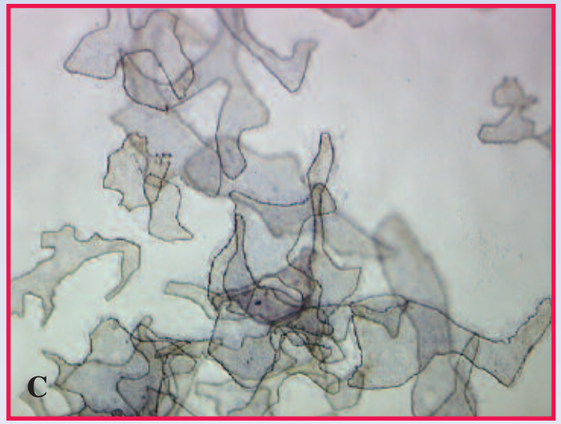
Microdissected material to be analyzed	Lysing buffer
Arcturus PixCell IIe (LCM)	Instrument gives approximate cell count. Tissue cut at 8-10 microns oftentimes is not a whole cell
Leica AS/LMD	Instrument gives area in micron-square totals



A. Before LM



B. After UV LM



C. LM samples in lysing buffer

Fig. 4. Frozen human lung sample stained per LM protocol. A) Target area imaged before laser microdissection. B) Target area cut out by UV laser. C) All targeted areas have collected in microcentrifuge cap containing 50 microliters of DNA lysing buffer.

area marked for microdissection. The second image depicts a serial section, briefly stained and uncovered, with the matching area before and after LM. These images, together with the molecular data, validate the study. Uncoverslipped, briefly stained slides display poor morphology but with practice and usage of landscaping techniques, the HT can mark the same area on all 3 images (map, before LM, and after LM) (Fig. 4). Both NIEHS instrument platforms allow images to be made of the microdissected sample removed from the tissue slide, which provides an informative presentation when coupled with the molecular data.

Optimizing each protocol step from collection through staining will allow the LM user to harvest the highest quality molecular target for subsequent analysis.

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Stained Slides vs Noninvasive Imaging: Strange Bedfellows in Bone Research?

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Introduction

Most veteran histologists have heard the following at one time or another: "You will be replaced by a machine; Histology will be supplanted by imaging modalities." In the clinical arena circa 1980, it was the advent of the cell sorter. In the 1990s in the orthopaedic research field, it was various evolutions of x-ray and computed tomography (CT). In each venue, however, quality histologic preparations are paramount for accurate disease diagnosis and the identification of cellular mechanisms.

Bone research combines aspects from a number of disciplines. Histology lends variants of paraffin microtomy and staining for decalcified bone and connective tissue, and polymer or resin microtomy for undecalcified bone. Systems for cutting and grinding of resin-embedded bone containing metallic implants have been developed for the study of tissue growth into and around orthopaedic prosthetic devices. The discipline of radiology contributes film, and more recently, digital single-plane x-rays. Body composition and bone mass are now measured with dual-energy x-ray absorptiometry (DEXA). Standard CT analysis has morphed into peripheral quantitative computed tomography (pQCT) and micro computed tomography (micro CT), allowing for the three-dimensional evaluation of bone in vivo or ex vivo. Positron emission tomography (PET) scan instruments are now being utilized in bone infection and metastasis studies. Micro MRI units are available in orthopaedic research core laboratories for imaging all aspects of the musculoskeletal system. More recently, in vivo imaging systems (IVIS) that image bioluminescence and fluorescence have become standard members of the orthopaedic research armada. In addition, biomechanics, the study of forces and strains on the development and structural integrity of bone, is still a prominent focus in orthopaedic research.

Bone is a complex tissue that undergoes remodeling throughout life in order to maintain skeletal integrity. As a result, bone can both adapt to altered loading and repair fractures. Defects in integrity may be expressed in terms of mineral density, cellular deficiency (either in numbers or function), or in combinations of these. It is this multiplicity of factors that makes bone research challenging. The biology of the osteoclast, or teams of osteoblasts, needs to be considered not only independently but also in relationship to one another, and relative to the physical load in an environment of specific biochemical and endocrine entities being produced by the body. The best studies will include many of the investigative modalities previously mentioned, employing the imaging devices as monitoring tools, and the histology for obtaining high resolution and mechanistic information. And in the end, the bottom line for all this remains: How can this information be used in the prevention of disease and improvement of quality of life for those already afflicted?

A number of important bone pathologies are characterized by loss of bone, either systemically, as in osteoporosis, or locally, as in periodontal bone loss, rheumatoid erosions of the joints, cancer-mediated bone destruction, and periprosthetic loosening. Conditions such as rheumatoid arthritis and osteoarthritis (OA) involve both bone and cartilage.[1] Intense research efforts have provided considerable insight into the mechanisms of bone turnover in health and disease, and have led to the identification of many of the molecules involved in these processes. However, it is clear that a more complete understanding of bone diseases, and the development of improved therapies, will require the coordinated use of all current experimental techniques.

Current Studies: Histology Is the Foundation

Basic science studies facilitate investigation into cell biology and sophisticated skeletal phenotyping, as well as conventional orthopaedic pursuits, including biomechanical evaluation of implant function.[2] Many of these in vivo studies utilize animal models, including osteomyelitis in rabbits[3] and distraction osteogenesis in rats and mice.[4,5] Combination studies with these platforms include antibiotic efficacy, drug therapies, therapeutic agent delivery, and implantation.[6]

Rats and mice provide an excellent platform for the study of basic bone growth, healing, and response to injury. The immunologic compatibility between human and murine specimens, combined with precision processing of bone samples, has fostered studies on the effects of growth factors, diet, and specific diseases on bone growth and healing. The noninvasive imaging-to-histology pathways in these studies have crossovers and evolutions that closely parallel human clinical diagnostic conduits.

After all, many of the basic studies are developed to answer questions spawned by events seen in the emergency room, operating room, and in other patient populations. If correlations cannot be made, then the value of the information gleaned at the laboratory bench may not have significance for the patient. The primary divergence in this correlative approach can be in the confirmation of effect. In basic research, once a modality's result is *surmised* by electronic imaging, it is optimal to *confirm* the imaging data via postmortem histology. Clinicians must have far more faith in electronic imaging and serum testing because in the clinical arena, postmortem histologic analysis would not be an acceptable option. Once the clinician is satisfied that a bone infection is eradicated based on results from serology, x-ray, CT, or MRI, therapeutic intervention can potentially be ceased. But in a laboratory evaluation of the efficacy of a new infection eradication regimen, investigation by x-ray, CT, and MRI should be backed up by histological assessment of the tissues to ensure that there are no harbors of bacterial sequestrum present, or that there is no incidental cellular modification as a result of the regimen. In the orthopaedic clinic, a DEXA scan can interpret relative bone density in the osteoporotic patient. In the basic study of osteoporosis prevention, the DEXA and CT scans can reveal which treatments promote superior bone density and quality.[1] But no imaging modality can provide the cellular ratios of osteoclasts vs osteoblasts, the rates of lamellar bone formation, or information on osteoblast and osteocyte apoptosis. For this, techniques such as a histologic workup comprising undecalcified slides stained with methyl methacrylate (MMA) trichrome and von Kossa, as well as unstained slides for dynamic tetracycline labeling analysis, are required.

Electronic Imaging Tools

A cabinet-style, closed system, variable kilovolt x-ray unit is a two-dimensional x-ray tool that provides, by today's standards, low resolution images of the relative density of the material within the specimen by using long exposure times to yield images of differing contrast.

DEXA is a two-dimensional x-ray–based method for measuring bone density. What sets DEXA apart from plain film x-rays is that DEXA technology can be used to quantitatively estimate the actual mineral content of tissues, thus providing a more accurate determination of bone density in vivo. For comparative purposes, longitudinal repeat measures of bone density are possible.[7]

Peripheral quantitative computerized tomography generates three-dimensional measurements of volumetric bone density in the same manner as a clinical CT scanner. The three-dimensional data acquired facilitate the calculation of bone volume by combining data from serial images down to 0.11 mm in thickness. Whereas the two-dimensional instruments can provide information about the presence or absence and density of bone healing post-trauma, the pQCT provides differential volume of cortical vs trabecular bone either in vivo or ex vivo. Due to its enhanced sensitivity, drug efficacy can be evaluated at earlier timepoints. This concept is becoming increasingly important as in vivo studies of experimental treatments are demanding shorter experimental turnaround and improved resolution.

Micro CT is a closed system unit similar in appearance to a closed system x-ray unit except that this instrument produces volumetric CT data from serial images down to 10 microns in thickness.[8] At this level of resolution, quantitative static histomorphometric indices yielding precise estimates of strength are possible. This is valuable to the orthopaedics researcher because these quantitative indices are achieved without processing or staining of MMA sections. The nondestructive micro CT methodology for the determination of strength translates to no more "bone breaking" on the MTS 858 Bionix materials test system (MTS Systems Corp., Eden Prairie, MN). With this technology, a rat tibia in an osteogenesis study can be evaluated for strength and then submitted for histological evaluation with all the cellular architecture preserved, which means that significantly fewer animals will be needed throughout the course of the study.

Figure 1 shows a radiograph (x-ray) demonstrating bone loss due to breast cancer metastasis (MDA-MET) in a murine model. Although the presence of bone resorption is evident by x-ray, visualization of the mechanism by which this occurs calls for a tartrate resistant acid phosphatase (TRAP) stain performed on decalcified paraffin sections. As shown at 40X, osteoclasts are recruited and activated by the tumor and are responsible for the bone destruction observed on x-ray.[9]

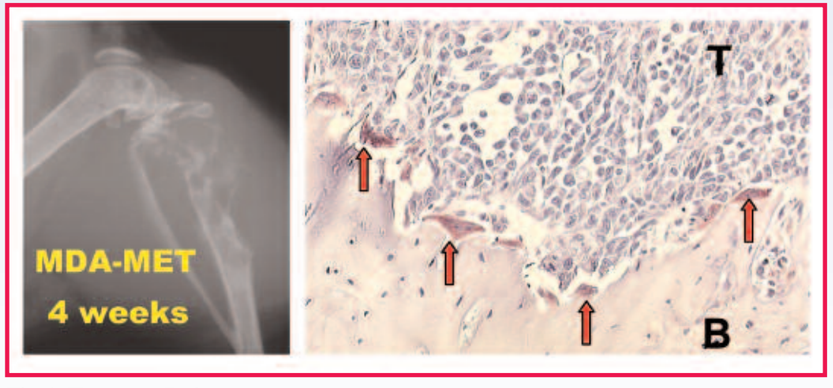

Fig. 1. T=tumor; B=bone.

Fig. 2.

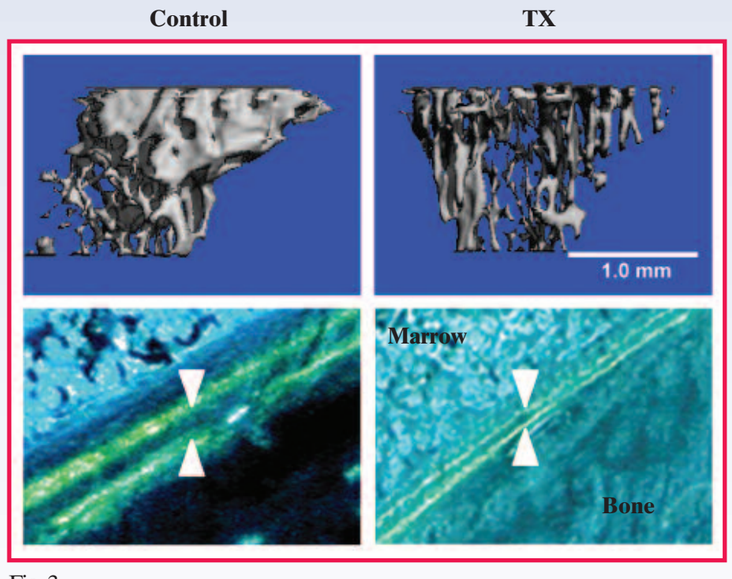

Fig. 3.

Figure 2 shows micro CT renderings of the tibiae of mice injected with tumor and treated with an experimental antibody treatment (Ab) or a control antibody (Control). While the micro CT image associated with Ab treatment shows maintenance of bone, the image of Control shows extensive bone destruction. However, the postmortem H&E straining of decalcified paraffin sections (Ab, 400X; Control, 100X) shows the presence of tumor (T) in both specimens that was not visualized by micro CT.

Figure 3 depicts micro CT renderings comparing bone micro architecture of a control mouse (Control) vs a mouse subjected to an experimental drug therapy (TX). While the images clearly depict a loss of bone as a result of the therapy, postmortem histologic analysis of a double-labeled single fluorochrome from undecalcified, MMA-embedded unstained sections indicates the mechanism of the bone loss; the primary reason for the lack of bone depicted by micro CT was not bone resorption as suspected, but rather suppression of normal bone formation[10] (arrows) (Control, 100X; TX, 40X).

Figure 4 shows TRAP-stained osteoclasts seen in an undecalcified MMA section of bone (400X). Specific labeling locates osteoclasts (arrow) in situ, and their location relative to the cortical bone interface helps assess activity associated with bone resorption analysis.

Figure 5 shows specific immunohistochemical staining for a human chondrocyte-specific protein in a decalcified, paraffin-embedded section of human bone (arrows) (100X). Proper fixation and precise endpoint decalcification allow for a wide range of IHC procedures.

Figure 6 shows tetracycline double labeling as viewed in an unstained section of undecalcified MMA-embedded murine bone (200X). Tetracycline is absorbed by newly forming bone at the time of administration. Two pulses of tetracycline at the appropriate times can assist in the evaluation of rates of mineral apposition and bone formation, calculated from the distance between the two labels (arrows).

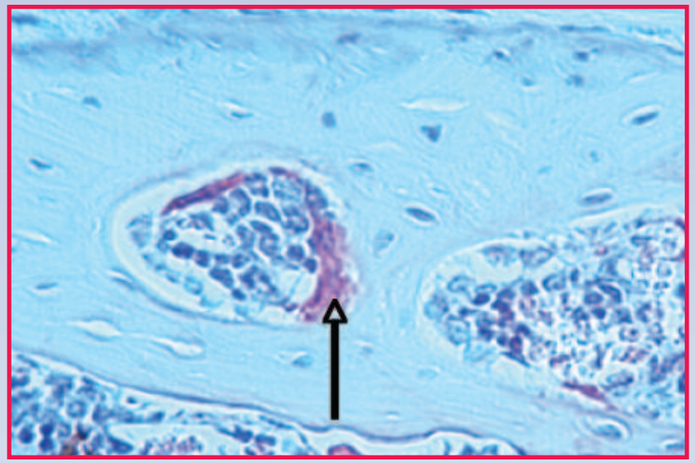

Fig. 4.

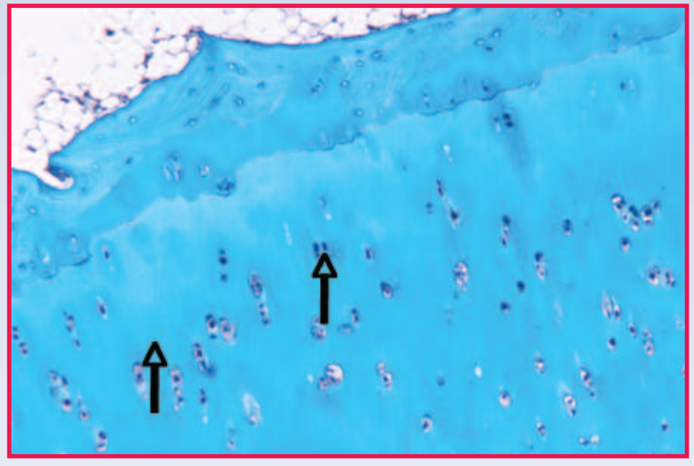

Fig. 5.

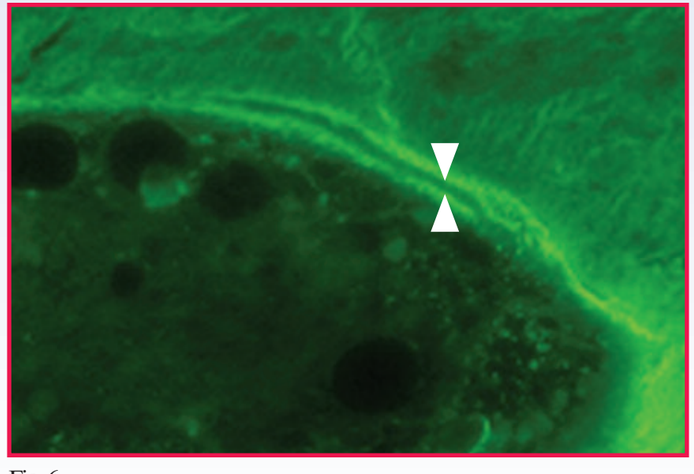

Fig. 6.

Discussion

So does this constantly evolving electronic imaging technology spell the end of histology in orthopaedic research? Not if there is a need for static and dynamic histomorphometric indices, double fluorochrome labeling, or bone cell histology. These techniques require a great deal of expertise and familiarity with a wide range of methodologies. For example, small samples derived from cartilage explants are processed through glycol methacrylate and sectioned and stained appropriately. Undecalcified samples containing metallic implants are embedded in specialized resins and submitted for "cut & grind" histologic preparation.[11] Throughout the myriad of studies in the orthopaedic research lab, precise decalcification, along with specific processing protocols and refinements in reagents and antibodies allow for application of antibodies such as VEGF (vascular endothelial growth factor), VEGF R1 and R2 (receptor 1 and 2), collagen types I, II, and III, BMPs (bone morphogenic proteins), and others. Apoptosis studies using TUNEL (terminal deoxynucleotidyl transferase biotin-d UTP nick end labeling) or caspase 3 staining are routinely performed in plastic, as well as studies involving RT-PCR and in situ techniques.[12]

There are numerous examples of the power of combinatorial experimental approaches that help to elucidate complex biological questions. We have used computed tomography to measure osteolysis around total hip replacement implants and its progression. Strong associations exist between the volume and progression of osteolysis and the polyethylene (PE) wear that occurs after implantation, suggesting that PE particles may be involved in bone loss. Tissue sampled from zones of osteolysis during implant revision surgery showed abundant PE particles, often within giant multinucleated cells. Immunohistochemistry and in situ hybridization demonstrated that cytokines that activate osteoclasts, such as receptor activator of NF-kappa B (RANK), RANK-ligand (RANKL), and tumor necrosis factor alpha (TNFα), were strongly expressed by large multinucleated cells containing PE debris.[13] A strong correlation was found between the following four parameters: volume of bone loss, PE particles, RANK

expression, and TNFα expression. Importantly, correlative in vitro studies revealed that RANKL and TNFα synergize to increase the volume of bone resorbed. This suggests that the interaction of TNFα and RANKL promotes osteoclast activity associated with polyethylene wear.[13] These (and other) data suggest that therapies targeting TNF activity may be useful in treating peri-implant osteolysis.[14]

Another example of a combinatorial approach is in experiments designed to elucidate the etiology of osteoarthritis. While this disease results in the destruction of articular cartilage, the subchondral cancellous bone also shows characteristic histological and histomorphometric changes. Using state-of-the-art molecular technologies, we have investigated the expression of skeletally active genes in OA.[15-17] Significantly, some of the changes in gene expression can be directly related to structural changes, suggesting important genetic regulation of the bone changes seen in OA. We are currently using a combination of genomic and proteomic approaches to further investigate the basis of this disease. These modern approaches directly relate to the tissue level changes, and have led to the development of a field that we have termed molecular histomorphometry. All histomorphometric indices are measured including osteoid, trabecular bone area, volume, forming and resorbing surface, and cell numbers. Several of these histomorphometric indices can also be determined using micro CT and appear to correlate well with direct histological measures. However, dynamic histomorphometric measurements are only possible from unstained fluorescent labeled plastic sections. Conventional histomorphometric data remain the gold standard for the assessment of the cellular mechanisms of bone turnover. It is the marriage of the two techniques that provides the complete picture of bone turnover.

The Future of Orthopaedic Research

This blend of new technology with established histologic procedures has produced a highly desirable situation for the basic research community. And there is a great deal more to be learned from the careful application of these approaches. For example, very few transgenic animals produced around the world have been examined with respect to their skeleton. If they have, it usually involves only a cursory glance at the gross anatomy. Another example is the study of metastatic cancer in bone and why it is so difficult to treat. It requires a much better understanding at the tissue and cell level regarding the establishment, proliferation, and osteolytic behavior of cancer cells in bone. The vision of developing these systems to the point where they are more, rather than less, involved in skeletal research, as well as an integral part of the clinical diagnostic repertoire, must be pursued. In some institutions where basic science-derived histology is a staple, the addition of new technology has actually increased histologic workload. The investigation of bone and musculoskeletal pathology, whether using animal models or in vitro approaches, combined with powerful imaging and molecular tools, must eventually be related to diseases of the human musculoskeletal system. Results from the potent experimental armamentarium we have at our disposal are beginning to answer questions about what *does* happen rather than what *may* happen. Many recent grant submissions involving the new technologies described here have ultimately hinged on the correlation of histologic data with the imaging data acquired. If this trend continues, with the introduction of new equipment and the development of new technologies, the role of the histotechnologist will be necessary and remain prominent.

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Orthochromatic and Metachromatic Action of Toluidine Blue: Application in the Staining of Mohs Micrographic Frozen Sections for the Detection of Basal Cell Carcinoma

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Abstract

Mohs micrographic surgery is a surgical technique designed to conserve normal tissue, which reduces patient disfigurement resulting from the surgical removal of skin cancers. This is accomplished by removing the tumor one layer at a time, after which the surgeon verifies microscopically if more tumor is present, necessitating the removal of an additional layer. In some instances, a patient's tumor may be so advanced that removal of surgical layers may continue for a day or more before the surgeon is certain that all of the tumor has been removed.

The Mohs surgery laboratory is required to rapidly prepare stained frozen sections from each layer of tissue submitted by the surgeon. This will allow the surgeon to examine the slides microscopically to determine if additional surgery is necessary. When a layer contains no tumor, the surgical margins are declared to be clear of tumor and no more surgery is required.

While some laboratories perform hematoxylin and eosin (H&E) staining of frozen section slides, others prefer to perform rapid metachromatic staining to reduce the time it takes to get results back to the surgeon. We examined the value of toluidine blue for the staining of Mohs frozen sections by demonstrating its orthochromatic and metachromatic properties when applied to skin samples containing basal cell carcinoma (BCC).

Materials and Methods

Tissue was surgically excised by the Mohs surgical method. Fresh, unfixed skin tissue was embedded and frozen in OCT™ compound (Sakura Finetek, Torrance, CA). Blocks were cut at 6 microns by frozen section method on a cryostat. Frozen sections were picked up on coated slides and placed on a slide warmer for 30 seconds to ensure good adhesion during the staining process and subsequently placed in 95% alcohol for fixation. Residual embedding medium compound was removed in a luke warm tap water rinse.

Slides were stained regressively in filtered 1% aqueous toluidine blue as follows[1]:

1. Fix slides in 95% alcohol – 1 minute
2. Rinse in warm tap water – several changes
3. Immerse in filtered 1% aqueous toluidine blue – 2 minutes
4. Rinse in tap water – 30 seconds
5. Rapidly differentiate in 2 changes of 95% alcohol using gentle agitation to partially decolorize (remove) excess stain
6. Dehydrate in 3 changes of 100% alcohol – 5 dips each
7. Clear in 2 changes of xylene substitute – 10 dips each
8. Coverslip and mount with permanent compatible mounting medium

Water and alcohol are the two solvents used most often to prepare dye solutions, each of which can remove dyes by means of physical action. The term differentiation refers to the partial removal of dye, which provides specificity of staining for different tissue structures. Following differentiation, the slides were cleared in a xylene substitute and mounted with a permanent mounting medium and coverglass.

Results

Microscopic examination revealed both micronodular and infiltrative BCC. Tissue components, nuclei, and islands of basal cell carcinoma retained the original orthochromatic blue color. The surrounding stroma, or connective tissue, stained a metachromatic pink/magenta color.[2]

We observed that toluidine blue is an effective and useful stain for Mohs surgery due to its unique property of staining basal cell carcinoma stroma red/purple. Islands of basal cell carcinoma retain the blue orthochromatic dye and the stroma is highlighted by the metachromatic reaction.

Discussion

Thiazines are excellent specific nuclear stains extracted by alcohol. Toluidine blue is a synthetic dye derived from substances found in coal tar. Coal tar dyes are typically soluble in both water and alcohol, but toluidine blue has a greater solubility in water than alcohol. Toluidine blue is a basic dye with metachromatic properties. Generally, it is employed as a nuclear stain and used regressively (overstained) and extracted rapidly in alcohol. In routine histology, it is used to stain amyloids and mast cells. Cell nuclei are acid in character. DNA, the major chemical component of the nucleus, contains metabolically inactive heterochromatin and is stainable with basic dyes. In

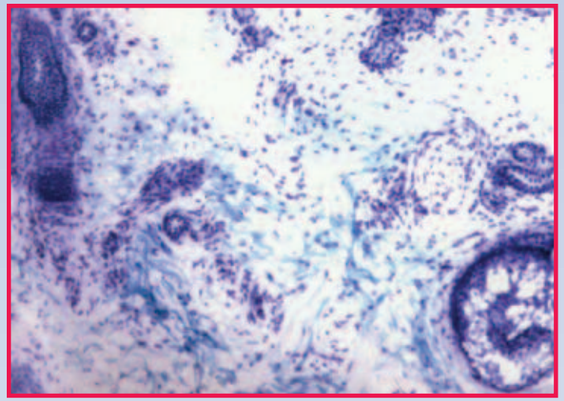


Fig. 1. Basal cell tissue components and nuclei stain orthochromatic blue. 100X

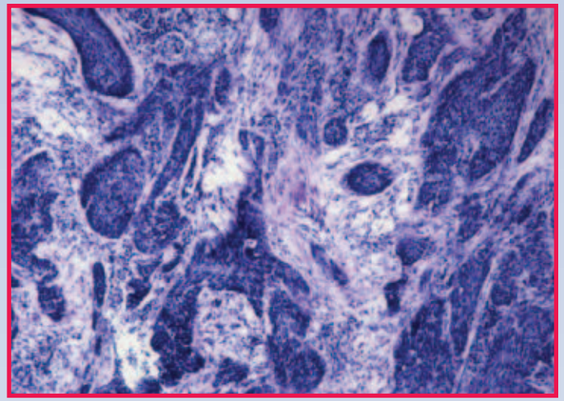


Fig. 2. Orthochromatic and metachromatic properties of toluidine blue demonstrating dense blue staining of BCC with pink highlighted stroma. 100X

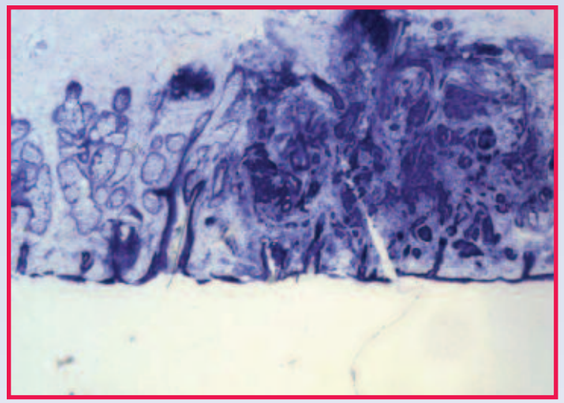


Fig. 3. Micronodular BCC with highlighted stroma. 20X

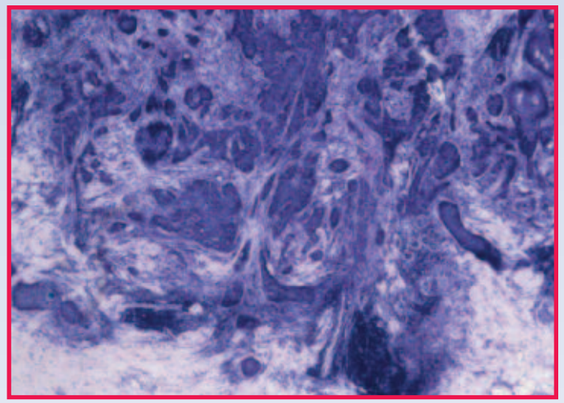


Fig. 4. Micronodular BCC with highlighted stroma. 100X

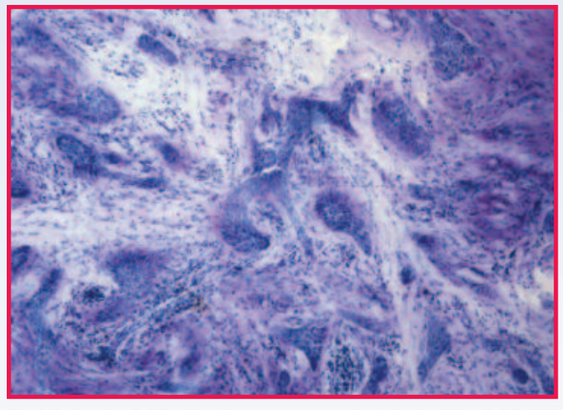


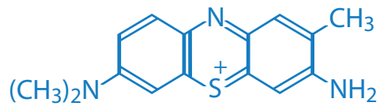
Fig. 5. Infiltrative BCC with pink stroma surrounding islands of blue BCC. 40X

malignancy, the nucleus undergoes changes, including increased size and irregular shape, hyperchromatic staining pattern, and an enlarged nucleolus.[2] Staining results associated with malignant cells are: cytoplasm and nissl substances—light blue; nuclei—deep purple; mast cells—red to deep violet; erythrocytes—green to pale yellow.[3]

Metachromasia (beyond color) refers to a shift in the color of a dye due to dimerization or binding to biopolymers. Charged elements within tissue alter the absorbed wavelength of visible light, changing the hue of the bound dye. Hues range from magenta to dark blue. A single dye may react with tissue components and stain each a color that is different from the original orthochromatic color of the dye. This selectivity may account for chemical factors while another part may be absorbed for physical reasons, such as density and surface area of the absorbing medium. If a dye is bonded physically to the tissue components, it is easily differentiated. However, a dye is not so readily removed when there is a chemical bond between a tissue and that dye. Metachromasia is thought to result from dense packing of the dye molecules to anionic sites on mucopolysaccharides. Basal cells produce mucinous stroma. Stroma, or connective tissue proper, may include fibroblasts, mast cells, histiocytes, adipose cells, reticular collagen and elastin, osteocytes, chondroblasts and chondrocytes, blood-thinning cells, and intracellular substances, eg, sulfated and nonsulfated mucopolysaccharides (carbohydrates).[4] Sulfated mucopolysaccharides are the most active naturally occurring substances that will stain metachromatically. Acid mucopolysaccharides stain pink. Therefore, islands of basal cell carcinoma retain the blue orthochromatic dye and the surrounding stroma highlights in a red metachromatic hue.[2]

Molecular Structure of Toluidine Blue[5,6]

Substances that stain metachromatically are sometimes called chromotropes and have a high molecular weight. The appearance of certain atomic groupings associated with benzene rings in a compound's structural formula may be associated with color and are called chromophores.[3] These include C = C; C = S; C = N; C = O; N = N; N = O, and NO_2. The greater the number of chromophore groups that appear in a compound's molecular formula, the more intense the color of that substance. In addition, color intensity increases as the number of hydrogen atoms increases. As a rule, yellow dyes are simpler in structure while red to violet and blue to green dyes are more chemically complex.



Common name	**Toluidine blue**
Suggested name	**Toluidine blue O**
C.I. Number	**52040**
C.I. Name	**Basic blue 17**
Dye Class	**Thiazine**
Ionization	**Basic**
Solubility in water	**3.82%**
Solubility in ethanol	**0.57%**
Absorption maximum	**620-622 nm**
Colour	**Blue**
Empirical formula	**$C_{15}H_{16}N_3SCl$**
Formula weight	**305.84**

Advantages of Toluidine Blue

1. Staining sequence requires fewer steps and less time.
2. Economical and space-saving.
3. The unique property of metachromasia imparts an identifiable staining pattern to basal cell carcinoma.

Disadvantages of Toluidine Blue

1. Lack of a counterstain for extracellular components.
2. The consistency of the stain is thick and requires coated slides and a slide warmer to prevent sections from floating off during the staining process.
3. A learning curve is expected to recognize unusual or difficult tumors.
4. Solution is not stable at a high pH of 10 and should be used fresh within a few hours of preparation.

Summary

Toluidine blue is a synthetic coal tar dye that is blue in color and acts as a base (cation) in solution. It is commonly used regressively and is useful for staining nuclei as well as mast cell granules and nissl substances. In general, it exhibits pink metachromatic staining in areas rich in anionic groupings in tissue (such as acid mucosubstances), allowing the bound dye molecules to interact with one another, forming dimers and polymers. In skin lesions it will stain basal cell carcinoma stroma a red/purple color, accentuating cytoplasmic differentiation with hues different from that of the orthochromatic color of the dye. Toluidine blue is an effective stain for Mohs frozen sections and is successfully used by many Mohs laboratories.

Acknowledgments

Thank you to Kishwer Nehal, MD and Yogesh Patel, MS.

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The Historical Evolution of Hematoxylin and Basic Fuchsin Staining of Biological Samples

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Introduction

Hematoxylin and basic fuchsin were two of the first dyes to be used for histological staining. Although both were originally used to color textile fibers, each made significant contributions to scientific progress after their adoption as biological stains. Despite having been in use for so long, hematoxylin and basic fuchsin are still two of the most commonly used biological dyes in the world today.[1,2]

The Need for Dyes in Tissue Science

Since the development of microscopy in the late 16th century, scientists have worked to improve the means of visualizing microscopic structures. However, the microscopic observation of biological specimens, especially the more transparent tissues and organisms, presented a few serious obstacles. It is difficult to see detail in many untreated specimens when using a light microscope and, even if detail can be made out, an observer may still have trouble discriminating different structures.

In 1719, microscopist Anton van Leeuwenhoek made a significant step toward resolving these issues when he colored his muscle tissue specimens with extract from the spice saffron in an attempt to disclose the cellular structure of the tissue.[3] According to his illustrations, this first attempt at tissue staining did not yield any substantial gain in visible tissue detail. However, the introduction of his idea—staining tissues with dye in order to enhance detail and distinguish discrete structures—set the stage for future work by other scientists. Only through the subsequent development and optimization of dyes and histological staining techniques did it become possible to reveal and study the minute structure of tissues.

Long before scientists began experimenting with tissue staining techniques, vast industries had already been developed around the science of staining textile fibers with a variety of natural dyes. Many of these dyes, including hematoxylin, were part of ancient or even prehistoric fiber processing methods that were imported to Europe as western nations built up their colonial empires.[4] It was with this array of mostly plant-derived colorants that histologists initially worked to illuminate the detail in cellular structures.[3]

Hematoxylin: Natural Dye With Staying Power

The history of hematoxylin staining dates back to its use in the pre-Columbian civilizations of Central America.[1] The Mayans and Aztecs used the dye extensively in the coloration of cotton and animal fiber. Hematoxylin is derived from the heartwood of the logwood tree, *Haematoxylin campechianum*. To use the dye, the Mayans boiled the heartwood in water to extract the hematoxylin and then let the solution oxidize with exposure to air. Tannins from the wood supplied iron, which acted as a mordant and allowed the dye to impart a rich, permanent black color.[5]

In 1502, only 10 years after Christopher Columbus reached San Salvador, the Spanish discovered the secret of the logwood tree.[1] They brought it to Spain where it was an immediate success, favored over indigenous botanical and lichen dyes because of its color-fastness and relative affordability. The popularity of the dye spread throughout Europe, but met with resistance in England where its use was outlawed due to claims of poor color quality. Apparently the Mayan mordanting process had not survived the trip, but the law was repealed almost 100 years later after English dyers adopted this technique.[4,5]

Meanwhile, the shipping trade in logwood was booming. Spain, and later England, set up logwood plantations around South and Central America and the Caribbean. In the 17th century, just one transatlantic shipment of logwood could sell for more than a year's worth of any other shipped cargo.[5] All this floating currency lured not only freelance pillagers, but also warring nations into the game of piracy.[1] For more than two centuries, logwood production dominated the Central-American colonies. Indian and African slave loggers, known as Baymen, moved tens of thousands of tons of logwood out of the Central-American and Caribbean swamps each year. By 1800, massive importation had flooded the European logwood market and hematoxylin became considerably less profitable.[5] Within 50 years, economical, vivid synthetic dyes were introduced, but as hematoxylin was losing favor as a textile dye, it was finding a new home in science.

In the 1830s and 1840s, scientists began experimenting with hematoxylin in staining tissue sections. In 1865, Franz Böhmer made a significant breakthrough when he combined a mordant with his hematoxylin after studying the fabric dyeing process. Later, researchers developed many different methods of staining with hematoxylin—modifying color and character of the stain by varying mordants and oxidation protocol.[6] Today, hematoxylin remains the most commonly used histological dye and it is the foundation of many tissue stains.[1]

Basic Fuchsin

The advent of synthetic dyes in the mid-1800s, spawned by Perkins' accidental discovery of the dye mauve,[7] drastically changed the textile industry and facilitated the development of novel histological staining techniques that would not have been possible using the previously available natural dyes.[3,8] In 1856, Natanson synthesized the magenta-colored dye basic fuchsin, also called rosaniline. The textile industry already produced satisfactory red colors using cochineal and madder dye, but the introduction of the synthetic fuchsin was important because it spawned the development of many synthetic rosaniline variants within a few years.[3]

This flood of new synthetic dyes was a boon for the variety-craving textile industry, but it also offered potential to histologists. In 1863, Waldeyer used a basic fuchsin variant in combination with blue and purple synthetic dyes and found that fuchsin would stain cytoplasm and nuclei differently. Basic fuchsin became important in organic chemistry with Schiff's formulation of the Schiff's reagent in 1866. This stain made it possible to detect aldehyde and carbonyl functional groups in solutions and in tissues. Böttcher pioneered alcohol differentiation following fuchsin staining in 1869, and Feulgen later extended Schiff's work by using Schiff's reagent to disclose DNA.[2,3]

Despite the serious public health scourges of the day, it is somewhat surprising that the use of dyes for staining biological specimens occurred first in tissues. Bacteria staining lagged behind histological applications, due in part to the late realization that these minute, microscopic entities were in fact involved in disease. The late 1800s saw the advent of techniques to stain the tubercle bacillus, the causative agent of tuberculosis, which even predated the work of Gram (1884).

From the early work of Paul Erhlich later modified by Franz Ziehl (1882) and even later by Frederick Neelsen (1883), basic fuchsin achieved acclaim as an essential dye for acid-fast staining. Today, the Ziehl-Neelsen stain remains largely unchanged from the original method, utilizing a solution of basic fuchsin with phenol (carbol fuchsin) to stain the organism's wall. This method is a well-known and important staple in the clinical laboratory's armamentarium for identifying infectious disease.

In contrast, today's Gram stain bears little resemblance to Gram's initial technique as he never utilized a counterstain and he didn't appreciate the significance that some bacteria did not stain with his method.[3] Today, we classify bacteria as Gram positive or Gram negative based upon an organism's ability to retain the dye crystal violet. This observation reflects differences in cell wall composition, which has implications for susceptibility to the different classes of antibiotics. Modern Gram staining techniques utilize basic fuchsin (or safranin) as a commonly used counterstain for Gram negative organisms.

The widespread use of basic fuchsin for the staining of carbohydrates, nucleic acids, and microorganisms makes it one of the most commonly used dyes in the histology laboratory today.

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Facts About Alcohol Dehydration of Tissue Samples

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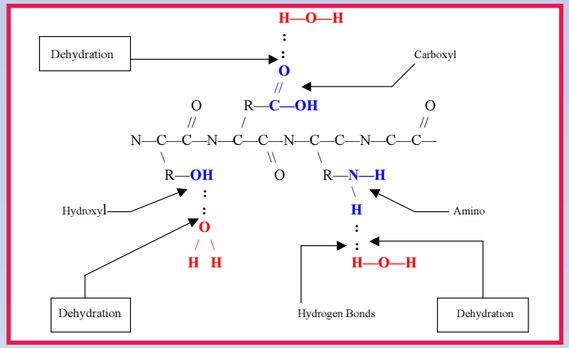
The dehydration of tissue samples in a processing cycle is defined as the gradual removal of water from interstitial spaces in the specimen; this is normally accomplished by a hydrophilic dehydrant such as alcohol. Before molten paraffin can be infiltrated in the tissue, water must be completely removed. Ethyl alcohol (ethanol) is the most commonly used dehydrant in tissue processing. Tissues are immersed in increasing concentrations of ethanol diluted in water, such as 50%, 95%, and 100% solutions. Ethanol is commercially supplied as absolute alcohol rectified to 96% or 98%; for practical purpose it is considered to be 100% alcohol.

The hydrophilic capabilities of a downgraded mixture of alcohol result in a slow and steady displacement of interstitial water preventing what is known as osmotic shock. Osmotic shock is the abrupt disruption of delicate tissue structures by the rush of poorly diluted alcohol into the sample. Integral tissue components such as connective tissue fibers and cellular components become distended or damaged by the effects of dehydration. In addition to the hydrophilic effect of alcohol in tissue dehydration, interstitial dehydration takes place by the physical mechanism of exchanged diffusion. Exchanged diffusion occurs when the concentration of water in one region is greater than in another. In our dehydration model, the higher concentration of water is found in the graded alcohol surrounding the tissue where the lower concentration of water is found.

There is another type of dehydration that is more harmful to tissue samples than the extirpation of interstitial water; it is the removal of structural bound water. Bound water molecules are normally attached to proteins by hydrogen bonds (hydrogen bonding occurs when covalently bonded hydrogen is attracted to atoms that have a strong electronegative charge) and are part of the structural configuration of the amino acids. Bound water is removed by alcohol during excessive dehydration and harsh and irreversible changes of tissue morphology result. Tissue samples become chalky white, dried, and unmanageable during microtomy. The collagenous portion of the tissue section acquires a glassy multidimensional appearance that is difficult to focus on under the high power of a light microscope.

In sum, the efficiency of dehydrating agents such as ethyl alcohol and isopropanol is directly proportional to their water binding capacity. These powerful hydrogen binding dehydrants compete with tissue molecules for the availability of water molecules, a contest usually credited to alcohol because of its efficiency in removing interstitial and bound water.



Bound water is integral in the steric interaction of the protein molecule. Water molecules are found attached to carboxyl (COOH), hydroxyl (OH), and amino (NH_2) groups in amino acids. Hydrogen bonds, denoted by ":" in the figure above, link water molecules to these three groups and are broken by excessive alcohol dehydration. These irreversible changes result in brittleness and alteration of the refractive index of the tissue, an artifact that can be seen microscopically as a glassy and multidimensional connective tissue.

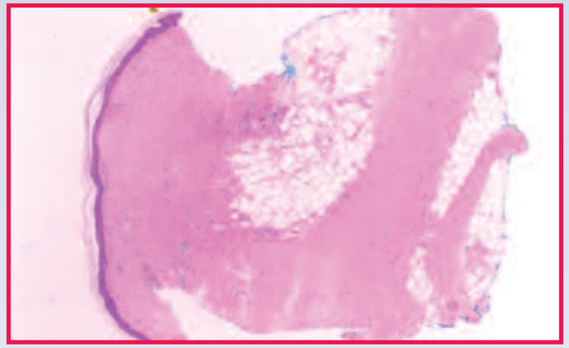


Fig. 1. Photomicrograph of a skin excisional biopsy. The dermal collagen, compacted and intact, is indicative of adequate dehydration during processing. The absence of over-dehydration artifact facilitates the pattern of analysis of the skin disorder. H & E stain, 20X

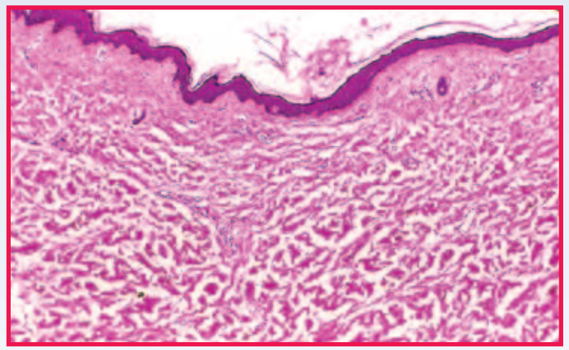


Fig. 2. Skin sample showing severely distended collagen in the dermal portion of the specimen due to excessive dehydration during processing. Notice the collagen bundles are coarse and glassy in appearance, difficult to focus under a light microscope; this artifact is the result of alterations in the refractive index of the tissue section. H&E, 100X

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Speaker: Sherri L. Anderson, HTL(ASCP)
Thermo Electron Corporation
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