The Impact of Study Design and Implementation on Outcome Quality

Robert A. Skinner, BS, HTL(ASCP)*; Dong Sun, MD†; William R. Hogue*,
Larry J. Suva, PhD*

* Department of Orthopaedic Surgery
  Center for Orthopaedic Research
  University of Arkansas for Medical Sciences
  Little Rock, AR

† Orthopedic Department of Southwest Hospital
  Third Military University
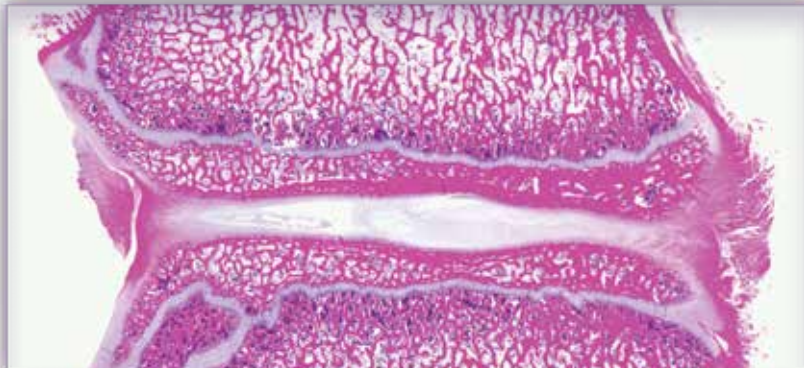 Chongqing, P.R. China

skinnerroberta@uams.edu

Fig. 1. 1367 x 1694 digital scan of H&E stained slide of vertebra-disc-vertebra composite section of spine from a porcine model of scoliosis surgery.
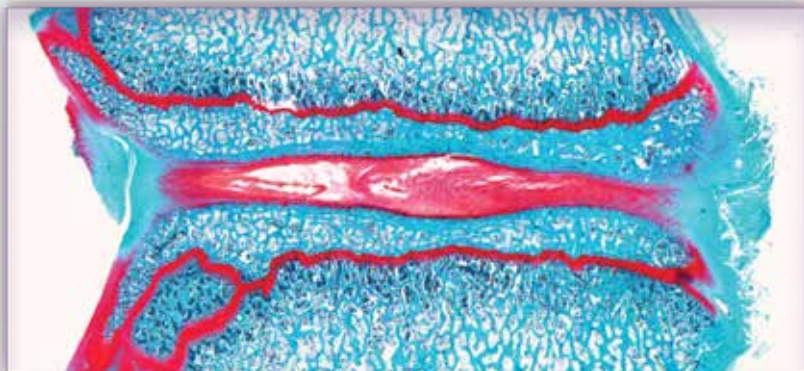

Fig. 2. 1367 x 1694 digital scan of safranin O-fast green stained slide from serial section of Fig. 1.

Abstract

The processing and sectioning of bone for publishable-quality slides often presents the histotechnologist with a number of interesting challenges. When you consider that histotechnologists have to create these slides from samples with the dimensions of a standard business card and the diameter of a toothpaste tube, it can be appreciated that the degree of difficulty grows exponentially. Add in the reality that the principal investigator's (PI) vision of what the final product should look like does not always align with the quality of the material presented at the grossing table and that, occasionally, all the presubmission planning in the world simply does not apply. At that point it may fall squarely on the shoulders of the histotechnologist to provide the steps necessary to salvage a study.

This report presents a research case review of a pilot study in which the PI misjudged the logistics involved in what was intended to be the optimal processing of pig vertebrae in a study of scoliosis. The ensuing dilemma presented the histologist with issues that were initially considered to be detrimental to the study but, in this case, had no significant bearing on the quality of the results.

Introduction

Over the past 40 years, members of what is now the University of Arkansas for Medical Sciences (UAMS) Center for Orthopaedic Research have employed concepts and procedures that have made the production of 2 x 3 inch slides from bone and joint samples routine. In brief, bone is cut down into 5 mm thick slabs and immersed in 10% neutral buffered formalin (NBF) as soon as possible following excision, no matter how large the specimen or what the particular tissue composition may be. From this point there is flexibility in being able to alter fixation time based on tissue density, with decalcification possible in the fluid of your choice. That's how things are in the ideal setting. However, what is ideal and what frequently happens are vastly variable. In fact, what is histologically ideal may not be investigationally ideal, and it is at this point where blocks and slides and papers and grants can be ruined.

Materials and Methods

The initial intent of this study was to use young mini pigs to investigate a surgical modality for the correction of idiopathic adolescent scoliosis. The mini pigs were projected to produce vertebral body specimens averaging ~1.5 to 1.8 cm in each dimension. They yielded specimens with block faces that would easily fit on a 1 x 3 inch standard slide. Even if a spine with 1.8 cm vertebrae was to be grossed to yield a block with an intact disc with trabecular margins of adjacent vertebra, that nominal 2.5 x 1.8 cm tissue face would still fit. The initial game plan was to isolate the target disc bookended by a vertebra on each side and bisect the construct longitudinally to facilitate optimal fixation and decalcification to produce a slide such as that depicted in Figs. 1 and 2.

Due to the PI's concern for the potential fragility of certain bone-to-soft-tissue interfaces in the region of interest, he requested that we not bisect his samples with our standard bone saw. This would require that we fix and decalcify the vertebral disc construct intact and then later bisect it with a disposable microtome blade prior to processing. As a result, consideration was given to the size of the specimen that would help achieve adequate fixation and decalcification. Our standard procedures are based on the Lillie postulate, which states that formalin penetrates normal tissue at a rate of 1 mm per 24 hours at ambient temperature and pressure.[1] Using this formula and thinking in 3-dimensional terms, it should thus take approximately 9 days to ensure complete penetration of 10% NBF. Having significant experience with a wide variety of bone sample sizes and densities, we were confident that this estimate was overly conservative and that these samples would in fact be completely fixed in 7 days. The slides generated from this initial run confirmed the accuracy of these projections.

Armed with the data gleaned from this initial run, the PI and surgical support team pressed forward with additional pigs. What was not anticipated by the histology team was that the PI had changed his study subjects from mini pigs to much larger standard pigs. In fact, the dissected spines were submitted as intact units that were placed in a 1 x 2.5 ft long plastic tub containing almost 10 gallons of formalin. The PI requested that the samples not be cut down further until after intact CT scans were performed (3 to 7 days post end-stage surgery). In addition, the PI wanted to analyze the scans (3 to 4 days post scan) before any grossing was performed. As a result, the vertebrae, which nominally measured 3 cm in any direction, remained as an intact construct (Fig. 3) of soft connective tissue of variable thickness and composition, which included cortical and cancellous bone and disc material each considerably thicker and more dense than the pilot mini pig samples. They were stored in formalin for a period of 6 to 11 days prior to being dissected.

Fig. 3. Postexcision photo of intact porcine spine before being placed for CT imaging.
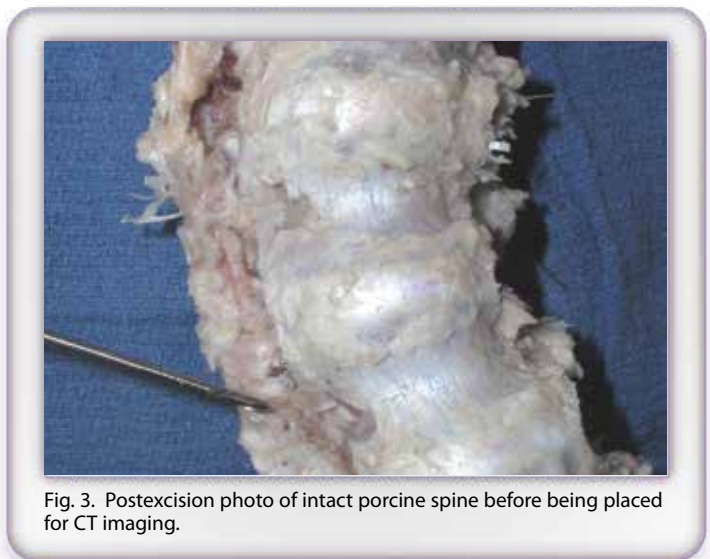
Samples containing the region of primary interest were initially cut down by sectioning through the discs of the two vertebrae adjacent to the specific disc to be studied. This ensured that the precise disc architecture would be maintained by preserving its relationship to the adjacent vertebrae. All samples were stored in formalin to achieve a target of 20 to 21 days of fixation prior to decalcification in 5% formic acid. The study sample was 30 mm thick and, according to Lillie's formalin fixation formula of 1 mm penetration per 24 hours, would require at least 15 days for formalin to reach the center. Given the thickness of the cortex and increased density of this particular construct, we proposed that a longer immersion time in formalin would be beneficial.

Decalcification was carried out in 5% formic acid that was changed twice daily for the first 2 weeks and once daily thereafter until the end point was reached as determined by the standard ammonium oxalate chemical test.[2] Samples earmarked for intact disc display were bisected through the center of the adjacent vertebral bodies parallel to the intact disc and subsequently cut across the disc, yielding blocks that would fit on our standard 2 x 3 inch slides (Figs. 1 and 2). We use the term "preliminarily indicated" in reference to end point achievement because, with some exceptionally thick specimens, a false-negative reading can

occur.[3] A small number of the 40 slabs that were generated for this study did produce this effect, which was detected when the whole vertebral body was bisected. These samples were returned to 5% formic acid until deemed clear (usually an extra 2 days).

Results

A small number of the bisected slabs contained a central focal area that displayed a color suggesting inadequate fixation prior to decalcification. The areas were of varying size but each, if present, was located in the center of the specimen. Because the 21 days in formalin was an educated guess and there was no way for us to confirm its completeness at the start of decalcification, we were now facing the possibility that we may have inadequately fixed the sample before decalcifying it. Not knowing if this was indeed the case, our initial response was to simply put these specimens back into formalin for 3 days prior to the start of our standard paraffin processing protocol.

The paraffin processing protocol employed for these samples is a longer extrapolation of our standard protocol for decalcified femoral head slabs, which utilizes methyl salicylate instead of xylene as the clearing agent.[4] The resulting blocks all sectioned well at 5 to 6 microns using a standard steel blade on a Lipshaw 80A manual sledge microtome. The resulting slides were stained with hematoxylin and eosin (H&E) and safranin O–fast green (SOFG) and analyzed for bone-to-cartilage architecture relative to changes induced by the surgical procedures employed as part of the expanded study.[5,6]

Fig. 4. H&E stained bone-cartilage interface illustrating cartilage remodeling or defect suspected to be in response to surgical procedure. 100X.
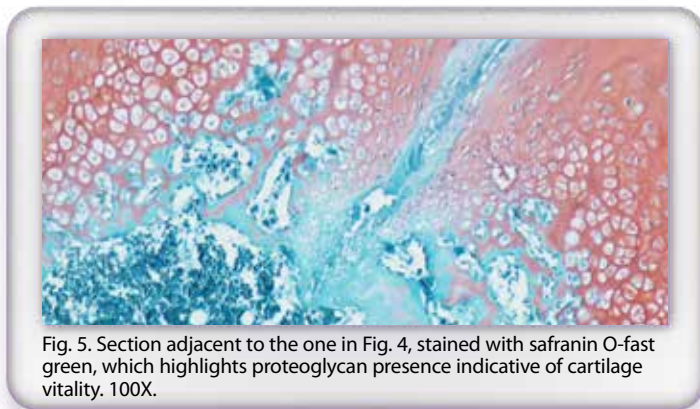

Fig. 5. Section adjacent to the one in Fig. 4, stained with safranin O-fast green, which highlights proteoglycan presence indicative of cartilage vitality. 100X.

Microscopic assessment of the stained slides confirmed quality material for evaluation of the changes associated with the mechanisms of the study. Variations within the architecture of the growth plate cartilage are clearly seen via both H&E and SOFG staining (Figs. 4 and 5). From a purely histologic perspective, there were certain manifestations that could have been improved, but in the context of the entire scenario we were more than satisfied with the results achieved, as was the PI. The midline area along the postdecalcification gross margin (Fig. 6) was thought to be the most central portion of the intact vertebral body and therefore considered to be either the last or the least fixed area of the bone. This photo clearly demonstrates discernible cellular details in the marrow along the marrow-trabecular interface.
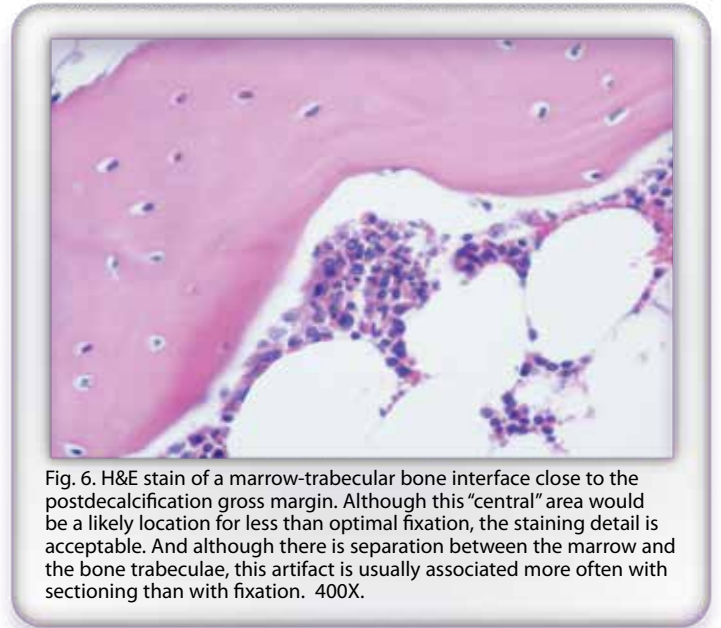

Fig. 6. H&E stain of a marrow-trabecular bone interface close to the postdecalcification gross margin. Although this "central" area would be a likely location for less than optimal fixation, the staining detail is acceptable. And although there is separation between the marrow and the bone trabeculae, this artifact is usually associated more often with sectioning than with fixation. 400X.

Cellular detail would be better if the sections were thinner, but this would most certainly be at the expense of otherwise overall intact architecture, as seen in Fig. 7.

We were also very surprised at the lack of structural integrity in the central areas of and along the disc (Fig. 8). Initially we thought this was a processing deficiency most likely associated with insufficient paraffin infiltration. However, after closer inspection of the cartilage-to-bone/bone marrow interfaces adjacent to this disc deficiency, we noticed a staining pattern (Fig. 9) similar to that in mouse bone, resulting from being inadvertently placed unfixed directly into 5% formic acid until end point. The marrow and trabecular material from this ill-fated mouse study sectioned well. By fine-tuning the H&E stain parameters, some architectural and cell population data could be achieved but, beyond that, little useful information could be derived due to weak and nondistinct staining. In this study report, thicker sectioning allowed this poorly fixed, weakly stained area to be more effectively visualized. In a study where the area of interest is in the outer edge of the vertebral body, detail such as that observed in Fig. 10 is sufficient despite the recognized histological shortcomings.

Fig. 7. H&E stained slide of outer third of cross-section of the disc between the vertebral bodies in Fig. 1. The overall section and stain quality indicate adequate penetration of the fixative and decalcifier necessary for a preparation of this type. 40X.
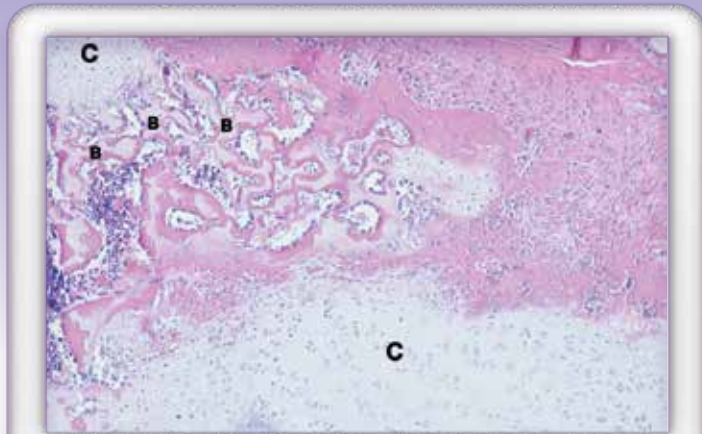

Fig. 10. An area of remodeling bone (B) between two fields of cartilage (C) displaying intact architecture and quality staining. H&E stain, 100X.
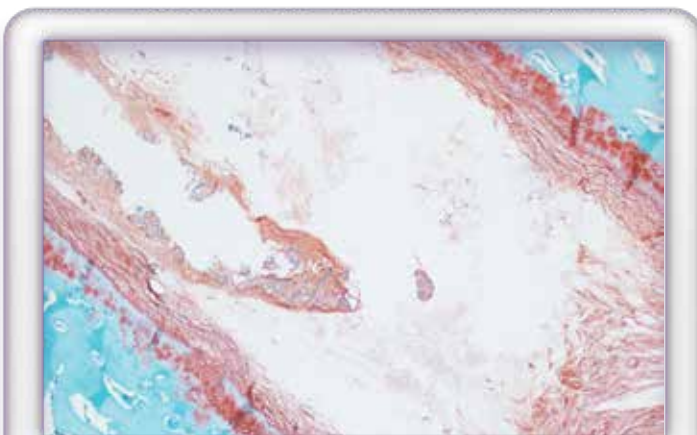

Fig. 8. Central-most area (inner third) of disc exhibiting poorly preserved architecture. Serial section of slide in Fig. 7, stained with safranin O-fast green. 40X.
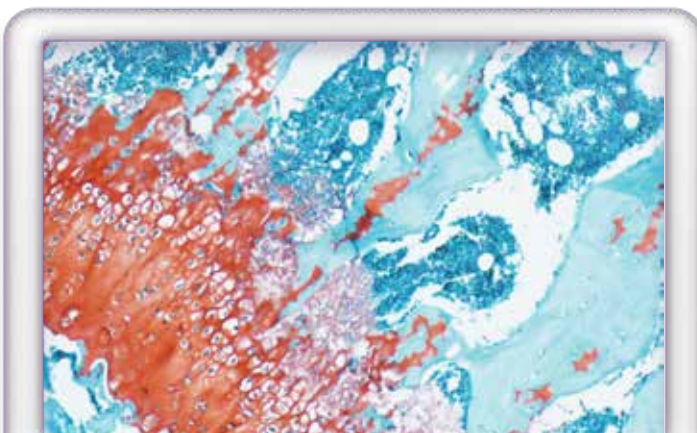

Fig. 9. Cartilage-trabecular bone-bone marrow interface in the vicinity of the poorly sectioned disc in Fig. 8. This field displays focal areas of nondescript weak staining in each tissue type for all safranin O-fast green staining components indicative of potentially poor penetration of the fixative. 100X.

Discussion

Methods for obtaining the best-quality slides from large decalcified bone samples are readily available in the literature and, when followed, can produce excellent results.[7] Occasionally, real-world laboratory situations, especially in a research setting, preclude the implementation of these methods. Ideally, the intact vertebrae in our study should have been dissected at the time of excision through the appropriate disc and then split with a saw, such as the EXAKT pathology saw (EXAKT Advanced Technologies, GmbH, Germany), which employs a diamond edge band saw blade and is suitable for handling samples of this size, which would have preserved the integrity of the interfaces. This would have allowed for more confident fixation.

Had the samples been sawed in this manner following initial fixation, we believe that greater slide quality would have been achieved. We have had extensive experience with resected femoral heads that were left intact for several weeks before being cut down. In that instance, red areas of suspected inadequate fixation were revealed when slabbed on a saw, so the slabs were returned to formalin before decalcification was initiated. This strategy succeeded in providing adequate microscopic results in past studies.

Based on our prior experience with femoral heads, we would not have been surprised to observe fixation-deficient staining. However, seeing this abnormality in the areas around the central portions of the specimen adjacent to the disc led us to reevaluate our time calculations for fixation of such composite samples. For composite specimens (bone + soft tissue with or without tumor), we traditionally base fixation time on the size and density of the cortical bone and/or articular cartilage interface. Because we perceived the density of the disc material to be less than that of the bone, we did not see the need to give the disc special consideration. In this instance it appears that more consideration should have been given to the chemical composition of the disc

rather than merely its size and density. The unique viscosity of the disc's "ground material," the presence of chondroitin sulfate, keratosulfate, and specific polysaccharides not found in bone or cartilage, as well as what is described as "disorderly collagen fibrils," suggest that vertebral disc may be an outlier when applying Lillie's formalin penetration formula.[8] Perhaps in samples such as these it is the disc that should be given primary consideration for fixative penetration estimates.

Conclusion

The appearance of insufficiently fixed areas of our study samples left us believing that the samples in this study were potentially ruined. From a microscopist's viewpoint, these preparations may not meet the microscopic criteria for publication-quality slides. Insufficient or delayed fixation can raise doubt about the validity of staining results in such instances. However, when viewed from the perspective of the orthopaedic surgeon/researcher whose primary interest is in early event remodeling, indicated by proteoglycan production in response to load, as applied in the surgical correction of scoliosis in a porcine model, the concept of publication-quality slides has an entirely different meaning. The slides produced have already provided a wealth of data both for the current study as well as for histotechnologists regarding how to handle these and potentially more challenging samples in the future.

While we were fortunate that our results were sufficient for the needs of the PI in this study, the circumstances described above illustrate how important it is that the specimen handling strategy be appropriately tailored from the outset to ensure that the desired outcome is achieved. The authors have shared this information not as an example of best practices but as an illustration of how even the best planning can go astray. The range of the histotechnologist's experience and knowledge is often essential to a successful overall outcome of translational biomedical research.

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Acknowledgments

An Unusual Presentation of *Histoplasma capsulatum* in the Colon: A Case Study

Kayla L. Weaver, MAT, PA-C

Athens Gastroenterology Association
Athens, GA

kweaver@athensgastro.com

Abstract

An adult male with symptoms of diarrhea and left lower-quadrant pain that had been intermittent for more than 2 years was being evaluated for possible Crohn's disease. Because of the severity of his symptoms and an abnormal CT scan demonstrating the presence of colitis and ileitis, he underwent a colonoscopy. Biopsy tissue taken during the colonoscopy identified the presence of colonic histoplasmosis. This report discusses the etiology of histoplasmosis and its presentation in an otherwise healthy male with no respiratory complaints.

Introduction

Histoplasmosis is the most common fungal disease in both healthy and immunocompromised patients, with several thousand people infected yearly[1]; however, it is more prevalent in immunocompromised patients. It is a pathogen endemic to the Ohio and Mississippi River Valleys in the United States as well as in Central America and Africa. Transmission occurs by inhaling spores or microconidia of the fungus *Histoplasma capsulatum*. The primary presentation is usually a pulmonary infection ranging from mild pneumonitis to severe respiratory distress syndrome. Although other manifestations are possible, it is a rare occurrence when *H capsulatum* is found only in the colon with no concomitant respiratory manifestations.[2]

Although the clinical manifestations of histoplasmosis are well described, a definitive diagnosis of histoplasmosis cannot be achieved on the basis of clinical information alone because there is significant overlap of histoplasmosis symptoms with those common to other diseases. The definitive diagnosis requires isolation of *H capsulatum* on specific culture media, or visualization of the yeast form in direct examination of clinical specimens using specific fungal staining techniques.

Discussion

A 37-year-old Chinese male patient presented with severe diarrhea. Imaging from a computed tomography (CT) scan showed abnormalities indicative of colitis and ileitis. The patient's symptoms had started 2 years earlier, but at that time his symptoms were mild. They had only become more severe over the past few months. A colonoscopy was performed to obtain biopsies. The colonoscopy showed the presence of congested mucosa in the terminal ileum, so a biopsy was taken of that tissue. Areas of inflammation were found in the colon 10 cm apart, and in the rectosigmoid colon, the sigmoid colon, and the descending, transverse, and ascending colon, as well as at the cecum, which was biopsied to rule out inflammatory bowel disease (IBS).

The patient presented for follow-up after the pathology report identified the presence of colonic histoplasmosis. He admitted that he had had a lung infection approximately 20 years ago while living in central China but denied having any current respiratory symptoms. He had lived along the Yangtze River where histoplasmosis is prevalent in China.[3] A diagnosis of histoplasmosis should be considered in patients who have relevant symptoms and have traveled or resided in China or the midwestern United States, Central America, or Africa.[3]
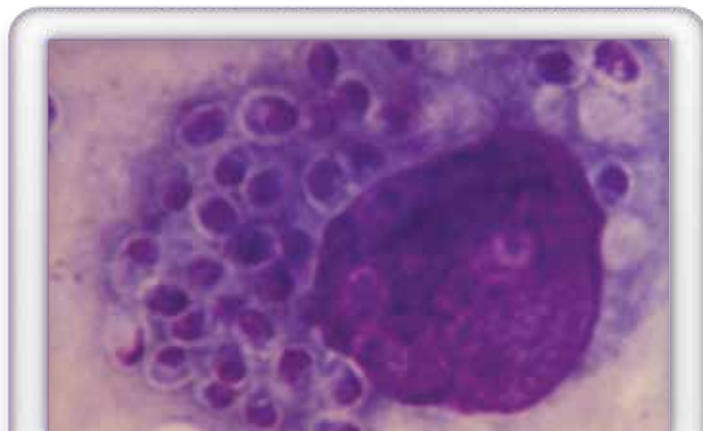
Fig. 1. Giemsa-stained photomicrograph revealing a histiocyte containing numerous H capsulatum fungal organisms in their yeast-stage of development. Image courtesy of Dr. J.T. McClellan, Lexington, KY, available at the CDC Public Health Image Library (PHIL) Web site.[4]

In hematoxylin and eosin (H&E) stained tissue sections, *H capsulatum* is seen as a small (1-3 microns in diameter) egg-shaped structure that has a basophilic center surrounded by faintly acidophilic material. The entire organism appears to have an unstained peripheral cytoplasm when stained with H&E. The organism may be difficult to discern and may occur extracellularly or intracellularly in aggregates of variable numbers in the cytoplasm of macrophages. The granulomatous lesions of *H capsulatum* can occur in various internal organs and tissues.[5]

A number of staining techniques may be utilized to demonstrate the *H capsulatum* organism (Fig. 1). Its wall contains polysaccharides that form reductive aldehydes upon oxidation that will bind Schiff stain; however, the periodic acid-Schiff (PAS) technique may not stain some nonviable fungal forms. Therefore, the modified Grocott-Gomori methenamine silver (GMS) technique is considered more reliable for general screening for fungus because it will stain both viable and nonviable fungal elements.

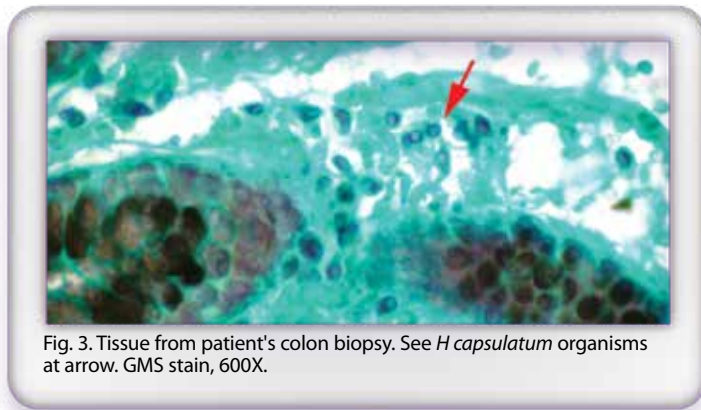Fig. 2. Tissue from patient's colon biopsy. GMS stain, 400X.

Fig. 3. Tissue from patient's colon biopsy. See *H capsulatum* organisms at arrow. GMS stain, 600X.

The use of the mild oxidizer periodic acid in the PAS stain results in the staining of many tissue structures that can distract from the small fungal organisms of *H capsulatum*. The method can be enhanced for fungi by utilizing chromic acid oxidizer in place of periodic acid. The GMS is probably the most widely used fungal stain, however, the somewhat capricious nature of silver staining may challenge even the most experienced histotechnologist. Insufficient silver impregnation, overstaining that results in high background staining, and undesirable precipitate may all contribute to difficulties in finding fungal organisms in stained tissue.[6] Histoplasmosis may cause extensive disease, yet, in rare cases, only a few organisms may be present. If the tissue is understained or overstained with silver, the few organisms present may be missed, leading to a false-negative diagnosis.[7]

Conclusion

A seemingly healthy patient who has traveled or lived in areas of geographical prominence for histoplasmosis and who presents with gastrointestinal symptoms should have an in-depth workup. It is uncommon for histoplasmosis to be found outside the lungs in a patient who is not immunocompromised; however, the patient followed in this case study illustrates that it *can* happen.

Histoplasmosis has traditionally been treated with the antifungal drug amphotericin B. However, amphotericin B is now used only to treat severe infection and only for a few weeks; it is then followed by azole therapy.[8] Itraconazole is the azole of choice following initial amphotericin B treatment for severe infection, and is the primary treatment used in cases of mild to moderate histoplasmosis.[9]

The patient in this case study was referred for pulmonary, ophthalmology, and infectious disease consultation after his histoplasmosis diagnosis. He started itraconazole therapy immediately and is currently under the care of an infectious disease specialist.

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New Guidelines for the Handling of Breast Cancer Samples

Vinnie Della Speranza, MS, HTL(ASCP)

Medical University of South Carolina
Charleston, SC

dellav@musc.edu

Pressure to provide a rapid diagnosis may force the surgical pathologist to take shortcuts in the grossing room in order to meet the turnaround time expectations of clinicians and nervous patients. This is actually so prevalent that pathologists have learned to overlook the morphologic artifacts that result when tissues have been inadequately preserved in formalin. In fact, some of these artifacts have become so commonplace that pathologists may forget to take into consideration that the microscopic changes can result directly from taking shortcuts in procedure and rushing the samples through the laboratory. The National Society for Histotechnology (NSH)/ College of American Pathologists (CAP) Histo-QIP™ proficiency program uses the term "incomplete fixation" to denote cellular changes that occur when incompletely fixed tissues are exposed to alcohol on the tissue processor. Artifacts may be of little significance to the surgical pathologist whose greatest concern is assessing if the tissue is adequate for use in making a diagnosis.

In this era of targeted cancer therapy, testing is now available to predict which treatment regimens are likely to benefit the patient. The pathology laboratory's role has expanded to not only provide a diagnosis, but also to make a patient's tissue available for molecular and immunohistochemical (IHC) analysis for markers that can determine if the tumor cells will respond to different treatment strategies. But the success of this type of testing rests largely on whether the patient's tissues have been well preserved, or fixed. After all, we can't expect to stain membrane markers successfully if their integrity was compromised at any point in the processing or fixation of the tissue.

Herein lies the conflict. Do we rush the laboratory procedures in preparing and processing the tissue so the patient can get a quick diagnosis, or do we slow things down to ensure well-preserved samples, which will increase the time the patient has to wait for results?

In 2003, an article by Goldstein et al entitled "Minimum Formalin Fixation Time for Consistent Estrogen Receptor Immunohistochemical Staining of Invasive Breast Carcinoma" gained the attention of the pathology community because it systematically examined IHC staining results for tissues fixed for various intervals of time, from a minimum of 3 hours to a maximum of 7 days. The authors concluded that IHC staining for estrogen receptor (ER) protein in their laboratory was optimized when the tissues were fixed in 10% neutral buffered formalin (NBF) for 6 to 8 hours "regardless of the type or size of specimen."[1] Fixation for less than 6 hours resulted in incorrect staining for this marker. The authors further noted that staining disparity between needle core biopsies and resection specimens from the same patient were likely the result of the shorter fixation times (less than 3 hours) utilized for core biopsies in their laboratory.
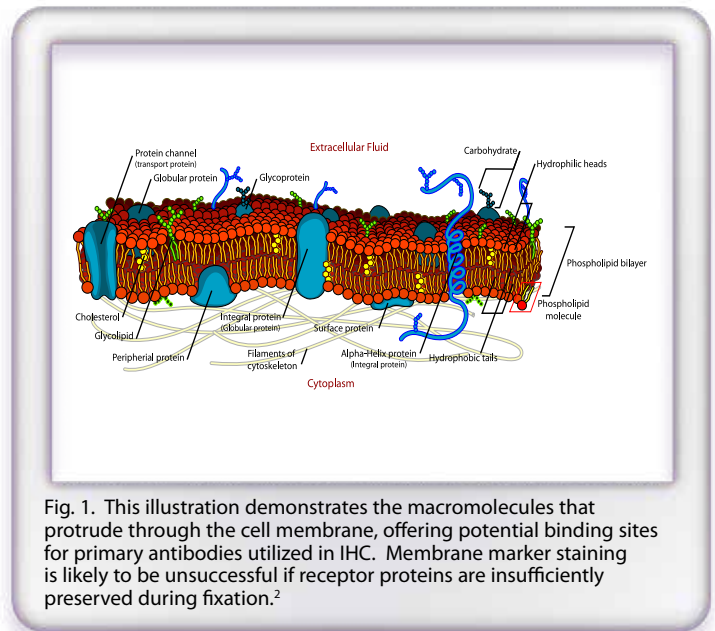

Fig. 1. This illustration demonstrates the macromolecules that protrude through the cell membrane, offering potential binding sites for primary antibodies utilized in IHC. Membrane marker staining is likely to be unsuccessful if receptor proteins are insufficiently preserved during fixation.[2]

Estrogen and progesterone exert their major physiologic effects in the breast at the cellular level by interacting with specific receptor proteins in the cell nucleus. Experimental, clinical, and epidemiologic evidence have confirmed that these hormones play a major role in the growth and differentiation of normal breast tissue and the development and progression of breast cancer.[3] It is well accepted that some breast tumors maintain their reliance on estrogen for growth and therefore estrogen blocking has offered an opportunity for controlling lesions in ER-positive patients.

For more than three decades, ER has been the most important biomarker measured for the management of breast cancer, largely because of the substantial benefit that endocrine therapy provides for ER-positive tumors in women of all ages. The clinical significance of ER has rendered the assessment of ER status of primary invasive breast cancer mandatory.[4] Endocrine therapy has been so effective that patients who have as few as 1% of their tumor cells positive for ER are offered this treatment option.
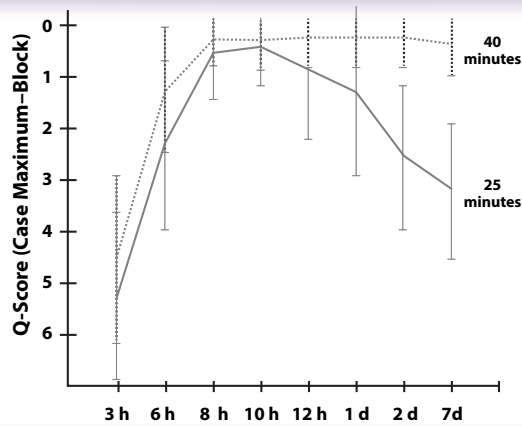
Fig. 2. Bernstein et al reported that maximum ER staining was achieved after 8 hours of fixation in formalin when epitope retrieval was carried out in EDTA buffer (pH 8) for 40 minutes at 95°C.[3]

Relationship of ER and PR to Histologic Subtypes of Mammary Carcinoma*

Type of Carcinoma	ER+	PR+
Infiltrating ductal, not otherwise specified (n=4,396)	3,255 (74)	2,330 (53)
Tubular(n=237)	237 (100)	225 (95)
Colloid (n=184)	184 (100)	133 (72)
Papillary (n=44)	44 (100)	35 (80)
Apocrine (n=40)	0 (0)	0 (0)
Medullary (n=96)	0 (0)	0 (0)
Metaplastic (n=120)	0 (0)	0 (0)
Infiltrating lobular (n=380)	380 (100)	293 (77)

ER = estrogen receptor; PR = progesterone receptor, + = positive.

*Data are given as number (percentage).

Fig. 3. Pattern of ER and PR staining reported in a study of 5993 breast cancers.[6]

Analysis of breast cancer tissue for estrogen receptor and progesterone receptor (PR) proteins has been the standard of care since the 1970s, initially performed as a biochemical ligand-binding assay, and later (mid 1990s) as an IHC stain. Consistent ER results are important because they are integral in making clinical therapeutic decisions. Incomplete preservation of breast tissue samples can result in false-negative staining, which would result in patients being denied endocrine therapies that might otherwise benefit them.

The human epidermal growth factor receptor 2 gene *ERBB2* (commonly referred to as *HER2*) is amplified in approximately 18% to 20% of breast cancers. Knowing one's HER2 status can be instrumental in determining treatment decisions. HER2 positivity is associated with worse prognosis (higher rate of recurrence and mortality) in patients with newly diagnosed breast cancer who do not receive any adjuvant systemic therapy. HER2 positivity appears to be associated with relative, but not absolute, resistance to endocrine therapies in general. HER2 status also appears to be predictive for either resistance or sensitivity to different types of chemotherapeutic agents. Perhaps most importantly, several studies have now shown that agents that target HER2 are remarkably effective in both the metastatic and adjuvant settings.

Trastuzumab (Herceptin®; Genentech, South San Francisco, CA), a humanized monoclonal antibody, improves response rates, time to progression, and even survival when used alone or added to chemotherapy in metastatic breast cancer. Other HER2-targeted drugs have been approved for the treatment of HER2-positive metastatic breast cancer.[5] It is generally accepted therefore, that HER2 testing should be routinely performed in patients with a new diagnosis of invasive breast cancer.

In 2007, a joint expert panel convened by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) met to develop guidelines for when and how to test for the HER2 gene, which at that time was reported to be amplified and/or overexpressed in approximately 15% to

20% of primary breast cancers. Since then, minor clarifications and updates to the ASCO/CAP HER2 testing guidelines have been issued, most recently in late 2013. These organizations also published guidelines in 2010 for IHC testing of ER and PR in breast cancer. Both the 2010 and 2013 publications contain essential data and recommendations for laboratories performing breast cancer pathology and associated marker staining.

In 2010, it was determined that up to 20% of ER and PR testing worldwide could be inaccurate (false positive or false negative), largely in part due to variation in preanalytic variables, thresholds for positivity, and interpretation criteria.[4] Similar to steroid receptor staining, there is legitimate concern about false-negative and false-positive HER2 assessments. A false-negative test result could lead to denial of trastuzumab treatment for a patient who could benefit from it. False-positive results could lead to the administration of potentially toxic, costly, and ineffective adjuvant HER2-targeted therapy. Both false-negative and false-positive results could be detrimental to the patient and must be considered and avoided by using best practices and the updated guidelines.

The 2013 updated guideline provides key information to pathologists and oncologists, with great emphasis on testing strategies, methodologies, and results interpretation. One change that is welcomed by histotechnologists is that the purported maximum formalin fixation time has been increased to 72 hours, which now matches the allowable maximum fixation time for ER and PR testing. Earlier stated maximums originally were set at 48 hours, which created operational challenges for laboratories not staffed on weekends.

Immunohistochemists have been and continue to be openly skeptical and critical of the current and previously published maximum fixation intervals (currently 72 hours in 10% formalin). Epitope retrieval techniques designed to unmask epitope binding sites have been employed by IHC laboratories for at least two decades. As a result, immunohistochemists believe that one cannot achieve false-negative staining due to overfixation,

assuming that one is utilizing a validated assay with epitope retrieval. Conversely, Bernstein's report clearly demonstrates that insufficient fixation has a dramatic effect leading to false-negative staining results.[3] It is important to emphasize that the guidelines for ER/PR (2010) and HER2 require that breast tissues receive a minimum of 6 hours in 10% formalin. Accredited laboratories are required to show evidence that this minimum is routinely met. Now that the maximum fixation interval has been increased to 72 hours for all breast markers, laboratories should have no difficulty remaining compliant with guideline recommendations.

But what about delays in fixation? Warm and cold ischemic times are widely accepted as important variables in the analysis of labile macromolecules such as proteins, RNA, and DNA from clinical tissue samples. Warm ischemic time is defined as the time from the interruption of blood supply to the tumor by the surgeon until excision of the tissue specimen; cold ischemic time is defined as the time from excision until the initiation of tissue fixation. The 2010 guideline discusses at length the need to standardize the cold ischemic time to no more than 1 hour.

Both the time of tissue collection (marked at the time that the tissue is removed from the surgical field) and the time the tissue is placed in fixative must be recorded on the tissue specimen requisition to document the time to fixation of the specimen.[4] Every effort should be made to expedite transport of breast excision specimens with a documented or suspected cancer from the operating room to the pathology laboratory as soon as they are available. It is quite likely that delayed fixation as well as incomplete fixation will contribute to erratic or erroneous results.

There is a misconception that smaller biopsy samples will fix more quickly than larger resection specimens and therefore require less time in formalin. Although formalin penetrates more quickly into these smaller samples, tissue fixation is a chemical reaction that requires time. As a result, small biopsy specimens require the same amount of fixation time as larger resection samples.[4]

The guidelines contain a great deal of information essential for every laboratory. The reader is encouraged to obtain the full text for both the 2010 and 2013 guidelines. The ER/PR guideline may be found at **http://www.asco.org/quality-guidelines/asco-cap-guideline-recommendations-immunohistochemical-testing-estrogen-and**; the HER2 guideline may be found at **http://jco.ascopubs.org/content/31/31/3997.long**.

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A Novel Method for the Storage of Unstained Tissue Sections

Jose A. Villada, HT(ASCP)QIHC;
Mohd K. Alfaraj, HTL(ASCP)QIHC;
George B. John, PhD, HTL(ASCP)QIHC

Department of Anatomic Pathology
Immunohistochemistry Laboratory
UT Southwestern Medical Center at Dallas
Dallas, TX

jvconsultinght@gmail.com

Abstract

Positive tissue controls play an essential role in the interpretation of immunohistochemistry (IHC) stains. Typically, sections to be used as positive controls are mounted onto glass microscope slides and stored at ambient temperature prior to use. The patient tissue to be studied may be mounted onto the same slide as the control tissue, or it may be mounted onto a separate slide.

One drawback of this method has been the gradual loss of antigenicity of the positive control sections that occurs over time when cut sections are stored at ambient temperature.[1] This problem forces laboratories to cut fewer sections at a time, which reduces storage time duration. Each time the control block is sectioned, the block face must be realigned, which results in unavoidable wasting of some of the precious control material. The antigenicity of the control section could be preserved if stored at low temperatures but previously frozen slides would be unsuitable for applying patient sections once the slide was thawed. A buildup of water condensate on the glass results in partial loss of charge that can cause loss of tissue adhesion, specimen degradation, and poor staining in both controls and patient tissue. Furthermore, having to prepare new slides from patient tissue blocks can lead to the loss of valuable patient specimen and less pathological tissue available for further diagnostic testing.

In a desire to avoid such difficulties, a new method was developed to store control sections. With this new method, unmounted control sections were stored in glossy fiberboard boxes under refrigeration. The control sections were later mounted onto glass slides alongside of the patient sections. The antigenicity of the controls was found to be well preserved for extended periods of storage with this method, resulting in sections exhibiting excellent staining quality. We noted that it took less time for a specimen to go from the lab to the pathologist, yielding an overall increase in productivity. This is both a cost-saving and time-saving method for preserving and storing unstained control sections in the immunohistochemistry laboratory.

Introduction

With the advances of new methods and technologies for the investigation of disease in organs and tissues, the preservation and indefinite storage of tissue samples for diagnosis or investigation with histological special stains, IHC stains, image analysis, molecular studies, and cytogenetic studies are crucial. Often such testing requires a combination of multiple techniques in order to obtain a valid and accurate diagnostic result. Therefore, it is imperative to avoid any loss of patient tissue specimens and positive tissue controls, and to preserve tissue antigenicity while trying to obtain optimum results.

The method reported here centers on the preservation of positive controls as individual paraffin sections without the use of glass slides. Tissue microarray (TMA) paraffin blocks containing about 4 to 7 cores (1 mm) of both positive and negative control tissue were used for this study.[2] The method was developed to enable placing the positive control and the patient sample on the same slide (positively charged slides). Previously, we had used control slides that were stored at -20°C, but noticed that tissue washed off of the control slides despite being on positively charged slides. This was presumably due to the accumulation of moisture on the glass slide that resulted in partial or complete loss of charge.

The new method involves precutting unmounted paraffin sections of positive controls and storing them in individual fiberboard boxes at 4°C until they could be mounted at the same time as the patient sample on glass microscope slides. With this method, the loss of tissue sections during washing was completely eliminated and the IHC staining quality could be assessed with confidence. Because this method allows for storage of many sections, it also decreases the number of times the TMA blocks have to be faced, thereby preserving the precious and limited control tissue.

Materials and Methods

Glossy fiberboard boxes 3 ½" x 3 ½" x 1" (Container Store, part # 162073), paraffin blocks, small desiccant bags, fine tweezers, and adhesive tape were utilized for this technique (Fig. 1). The paraffin blocks had TMA cores typically lined in one row. The blocks were melted at the upper vertical corners to shorten the length at the top so the section would have less resistance upon separation, yielding a better quality section (Fig. 2).

Tissues were chemically processed and embedded into paraffin. Sections were cut using a standard rotary microtome with low-profile disposable blades. The blocks were chilled and hydrated in a frozen ice bath for 5 minutes prior to sectioning in order to aid the formation of the ribbons. A 4" x 6" strip of thick black cardstock paper was affixed under the microtome blade holder to serve as an extended platform to support the paraffin ribbons as they were cut and to aid in separating the individual sections. The black background provides better contrast for visualizing the white strip of sections (Fig. 3).

Paraffin ribbons were cut at 4 to 5 microns thick, each containing 15 sections. Then the ribbons were separated into individual sections using curved-tip forceps and dissecting picks (Fig. 4).[3] The paraffin sections were then arranged systematically in white glossy fiberboard boxes on a piece of thick black cardstock, which makes the paraffin sections clearly visible (Figs. 5, 6).

Fig. 1. Materials used in this tissue storage method include glossy fiberboard boxes, TMA blocks, desiccant packets, labels, absorbent pads, forceps, needle probe, and adhesive tape.

Fig. 2. Close-up view of the typical positive control TMA block with 4 to 5 cores. The chipped edge on the top corner helps facilitate separation of the sections from the ribbon.

Fig. 3. A ribbon as it is being cut from the paraffin block, which contains many sections that will be separated into individual sections for storage. Black cardstock is taped to the microtome, providing support for the ribbon as it is cut while also offering good contrast to the white paraffin, making it easier to visualize the ribbon and its many sections.
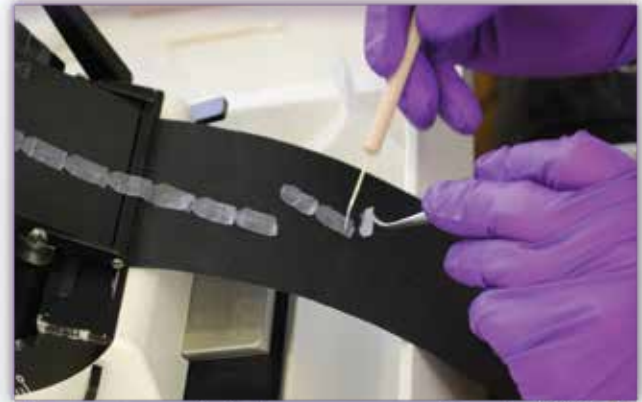

Fig. 4. The individual sections are separated from each other using a forceps and needle. Care is taken to avoid any tearing or physical damage to the sections.
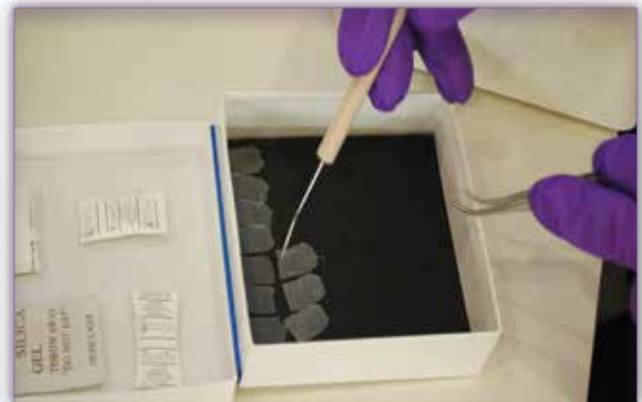

Fig. 5. Individual paraffin sections are placed sequentially in a row, avoiding tears or folding as they are placed in the bottom of the box on a piece of black cardstock.

Approximately 250 2-piece white glossy fiberboard boxes 3.5" x 3.5" were prepared. The lids were taped on the back to keep the lid and base unit together with an easy open-and-close motion. The boxes were identified on both the top and the lower front with the antibody names, control numbers, and the number of tissue cores in each block (Fig. 7) and that were verified by the TMA map in both the paper file and in the database. In addition, the first and last sections cut were placed in a water bath (38ºC-40ºC) to verify that all cores were represented and that after sectioning, the blocks were complete and would be useful for future sectioning.

A label maker was used to prepare the letters and numbers for box identification. Black cardstock paper lined the inside of the bottom of the box to more easily visualize the white or blue paraffin sections. The separated sections that had come from the same block were then placed adjacent inside each box. Depending on the size of the paraffin block, we were able to accommodate up to 45 sections per box arranged from top to bottom in a linear arrangement. The boxes containing the cut paraffin sections were stored in a refrigerator (4ºC-8ºC) to maximize antigenicity of the cut sections (Fig. 8). Vigorous or harsh handling of the boxes was avoided to ensure that the stored sections remained in the order they were placed into the box.

Fig. 6. Sections are arranged flat and close to each other, in the order in which they were cut, to maximize the number of sections in each box. Desiccant packets are taped to the inside of the lid of each box to help eliminate moisture during storage.

Fig. 8. The storage drawers are stacked vertically in a single-door refrigerator (4ºC), allowing for storage and easy access of more than 275 boxes.

Fig. 7. The glossy fiberboard boxes are labeled and placed in clear plastic storage drawers that hold approximately 12 boxes and allow for easy identification of each box. The boxes lie flat and are easy to retrieve and return to the plastic containers.

Fig. 9. A typical slide containing both control and patient tissue on a glass slide.

Discussion

This new storage method makes it easy to access and retrieve positive control sections from the fiberboard storage boxes when only individual positive controls are needed. It was easy to pick an individual positive control from the storage box and float it on the flotation water bath before mounting and placing it onto a glass microscope slide (Fig. 9). The control sections did not accumulate any visible moisture during the long periods of storage, and they did not move or get displaced within the boxes while the boxes were moved in and out of the refrigerator multiple times daily. The antigenicity of the stored sections was well preserved as demonstrated by the excellent staining quality we obtained on all of the antibodies tested (Figs. 10-26). We have successfully implemented this method for all of our clinical and research and development stains using both IHC and in situ hybridization (ISH) staining techniques.
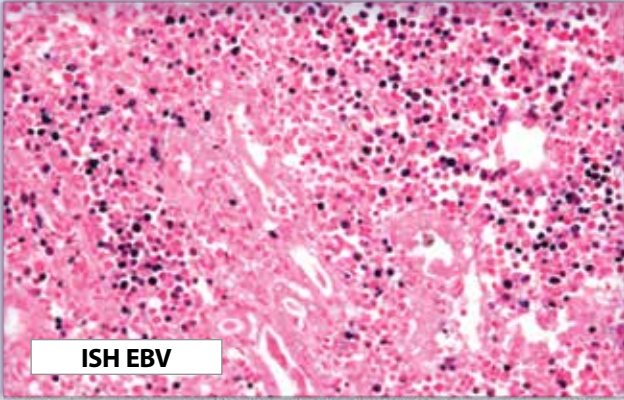
ISH EBV

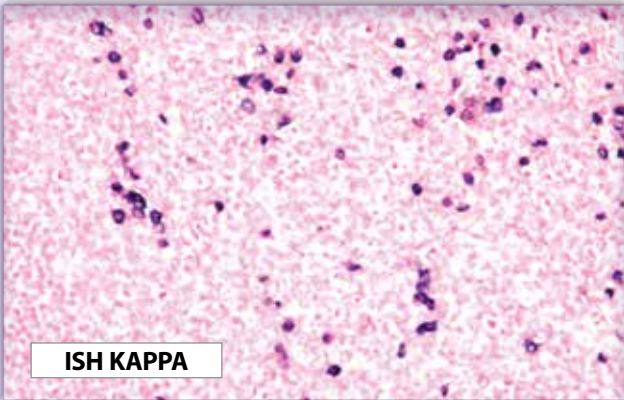Fig. 10. Epstein-Barr virus detected in lymph node by ISH. 100X

ISH HPV

Fig. 13. Human papilloma virus (low risk) demonstrated by ISH in an infected vocal cord. 200X

ISH KAPPA

Fig. 11. Kappa light chain present in plasma cells detected in lymph node tissue by ISH. 100X
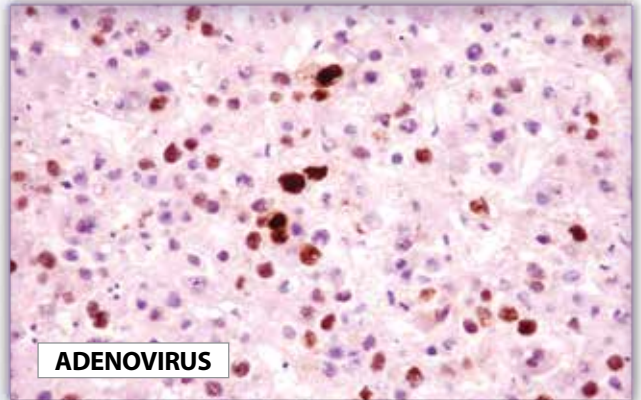
ADENOVIRUS

Fig. 14. Adenovirus-infected liver tissue demonstrated by immunohistochemistry (IHC). 200X

CD3

Fig. 12. CD3 highlighting T cells in the mantle and follicle of tonsil. 100X

HSV 1/2
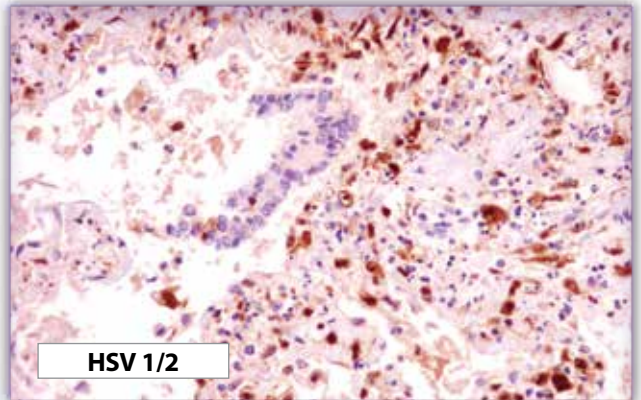
Fig. 15. Detection of herpes simplex cocktail in lung tissue by IHC. 100X

NEUROPATH
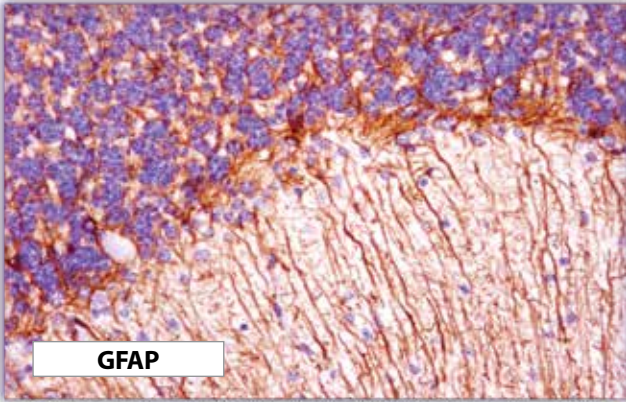
GFAP

Fig. 16. Glial fibrillary acidic protein (GFAP) demonstrated by IHC in cerebellum. 200X
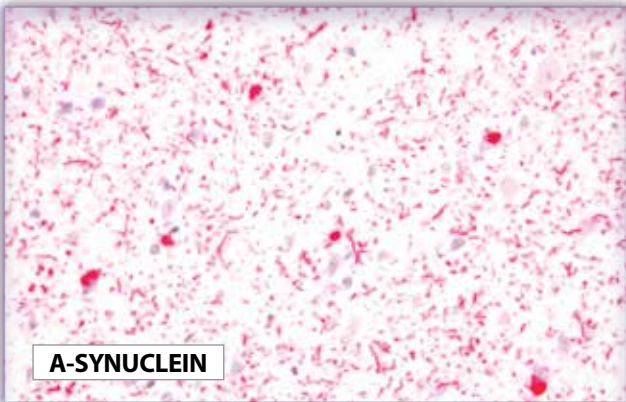
A-SYNUCLEIN

Fig. 17. Lewy body disease detected by positive alpha-synuclein staining by IHC in brain tissue. 100X
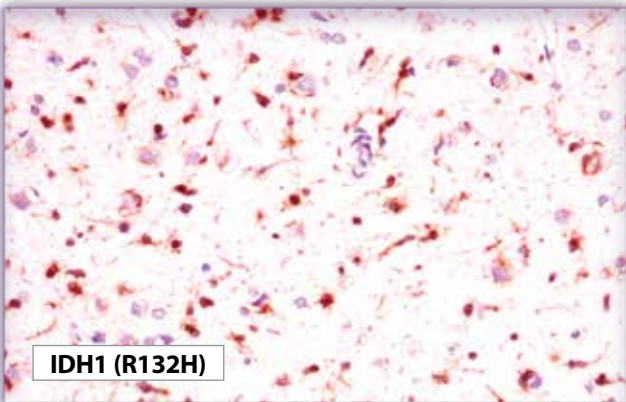
IDH1 (R132H)

Fig. 18. IDH1 (R132H) staining by IHC of a glioblastoma identifies the arginine to histidine mutated protein. 200X

CYTOKERATINS
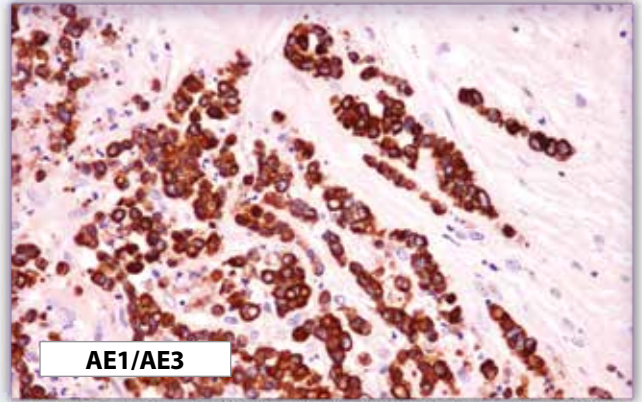
AE1/AE3

Fig. 19. Pancytokeratin AE1/AE3 in ovarian carcinoma by IHC. 200X
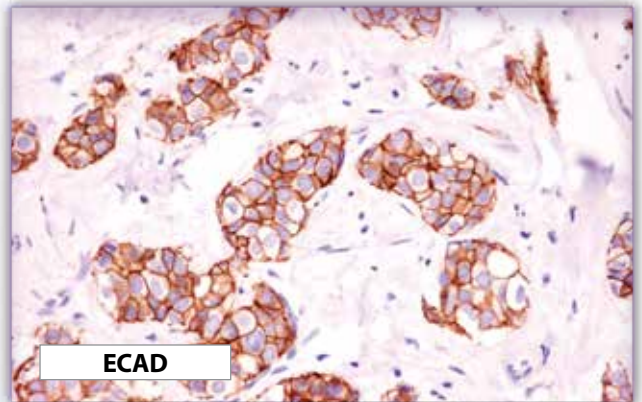
ECAD

Fig. 20. Positive staining of breast ductal carcinoma for E-cadherin (ECAD) by IHC. 200X

MNF-116

Fig. 21. MNF-116 staining demonstrates the glandular epithelial cells in prostate tissue. 200X

PIN-4

Fig. 22. Dual IHC staining using the PIN-4 cocktail (p63, HMWCK, and Racemase) to demonstrate prostate carcinoma. 100X
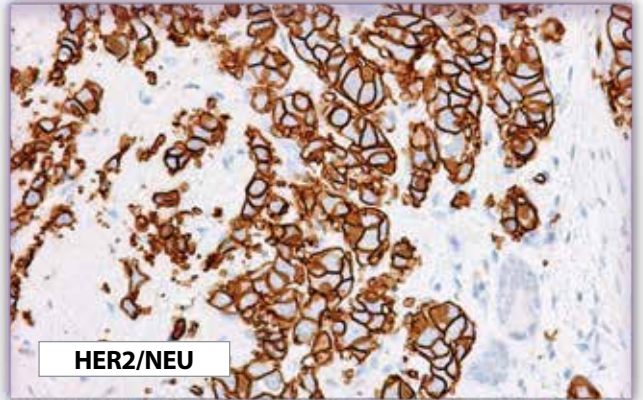
HER2/NEU

Fig. 24. IHC detection of HER2/neu protein amplification in breast tumor. 200X
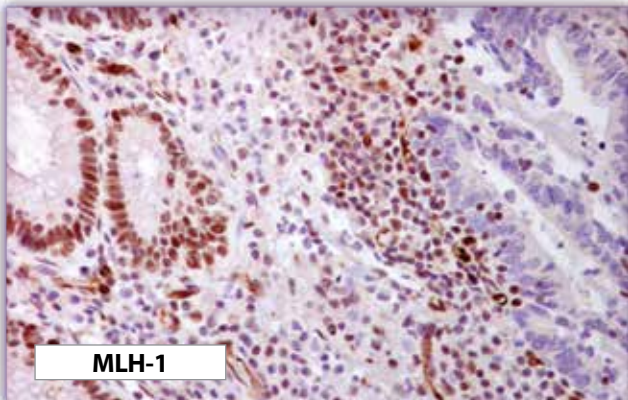
MLH-1

Fig. 23. Loss of MLH-1 in colon carcinoma cells detected by IHC in a case of Lynch syndrome. 100X
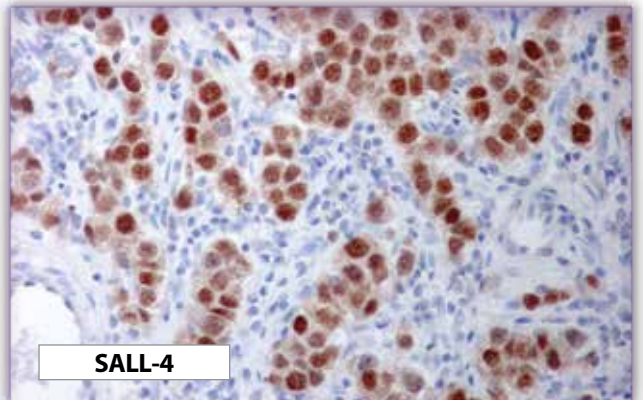
SALL-4

Fig. 25. Sall-4 marks the germ cells in a case of dysgerminoma. 200X

Conclusion

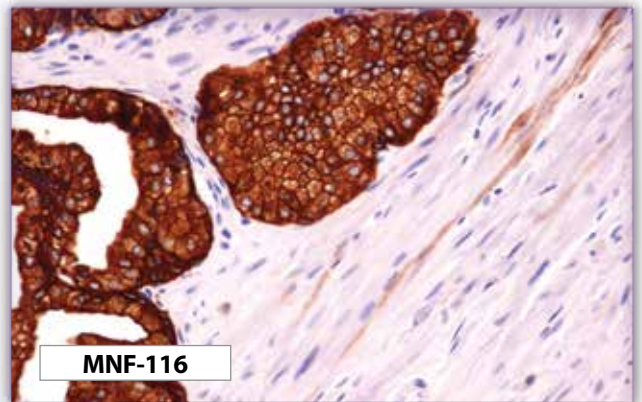Optimal preservation of unstained tissue sections can be achieved with this new storage method as we determined from the excellent staining quality we achieved with sections stored in this manner. This method is economical and it improved productivity in our laboratory by eliminating section wash off and providing enhanced antigenicity in stored tissue sections. It allowed us to avoid wasting expensive microscope slides (only those sections to be stained were mounted onto slides) and, most importantly, it helped ensure preservation of precious irreplaceable patient tissues that risk being lost if stored glass slides are ever broken.

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Acknowledgments

Localization of a Specific Region of Interest (ROI) in Different Tissue Sections on Multiple Slides

Salah Deeb, PhD; Mahmoud El-Begawey, PhD; Khalid A. El-Nesr, PhD; Shimma El-Nahas, PhD

Excellence Center of Pathobiology
Faculty of Veterinary Medicine
Beni-Suef University
Beni-Suef, Egypt

khnesr@yahoo.com

Abstract

A simple and efficient method for localization of the region of interest (ROI) in multiple slides is described. The method was carried out using a marked glass slide as a mask and was verified in slides stained with hematoxylin and eosin (H&E) and immunohistochemical (IHC) stains for detection of breast cancer surface markers estrogen receptor protein (ER) and progesterone receptor protein (PR), as well as human epidermal growth factor receptor 2 (HER2) and Ki-67.

Introduction

 A region of interest (ROI) is a specific portion of an image on a slide that will be used as a filter or for further investigation. We define the area in an ROI by creating a binary mask, which is a binary image the same size as the image we want to process with pixels that define the ROI. This may be used in cases to study tumor markers,[1] for prognosis and prediction of tumors by detection of metastasis (eg, breast tumors),[2-6] as well as in the study of neoangiogenesis of prostate tumors[7] and localization of cell nuclei. Breast cancer aggressiveness can be correlated with proliferation status of tumor cells, which can be ascertained with tumor grade and Ki-67 indexing.[8]

Optical microscopes are usually equipped with a scale as a part of the microscope stage. The scale can be used to locate any planar dimension in a microscope field because the ocular can be turned in any direction and the object of interest can be repositioned with the stage manipulators. To locate an object on a slide, one must note the number on the x- and y-axis on both micrometer scales. This can easily be done for a certain object on the same slide, but it is difficult to localize the same object in different sections mounted on multiple slides because it can be problematic to keep the position fixed to the same place along the glass slide.

We investigated the ability to take a captured image with a specific ROI, stained with H&E, for example, and transfer it to a newly acquired image of the same section stained with a different stain on a different slide. This method can also be useful in sections that have been destained and restained. We verified this method with slides of breast cancer stained with H&E and IHC staining for detection of estrogen receptors, progesterone receptors, HER2, and Ki-67.

We were also able to locate and define more than one ROI in an image, including regions that were irregularly shaped or geographic in nature, such as polygons.

Materials and Methods

A clean glass slide stained with hematoxlin and eosin (H&E) was used as the master slide, and 4 cut sections of mammary gland from a case of breast cancer were stained for expression of surface markers ER and PR, HER2, and Ki-67, which were used as positive controls. The breast lesion was diagnosed pathologically as ductal carcinoma with cribriform pattern.

Case history

The lab received a modified mastectomy specimen with an ellipse of skin containing the nipple and areola, which showed no surface ulceration. The whole specimen measured 26 x 15 x 5 cm and the skin ellipse measured 25 x 9 cm long. Upon sectioning, a firm grey-white mass measuring 2.5 x 2 cm was seen along with another multifocal tumor 1.5 x 1.0 cm away from the first tumor. An axillary fat pad was also received, from which 17 lymph nodes were trimmed.

Microscopically, sections contained breast tissue demonstrating ductal carcinoma in situ (DCIS); the infiltrating tumor was formed of moderately pleomorphic small cells showing moderate attempts at tubular formation and very low mitotic index. There was marked desmoplasia (the formation of fibrous connective tissue), and the intraductal component was primarily of cribriform pattern, with solid and comedo (indicative of necrosis) pattern representing 25% of the tumor. No lymphovascular emboli were seen. Sections from nipple and areola were unremarkable. Surgical margins were free of tumor, including the deep margin. Sections from the 17 enlarged lymph nodes showed metastatic deposits (4/16) with capsular rupture. After microscopic evaluation, the lesion was typed and graded as multifocal infiltrating ductal carcinoma grade 2, with in situ elements 25%, free margins, and positive nodal metastasis (4/16).

The breast tissue specimen was routinely processed with H&E stain. Estrogen/progesterone receptor (ER/PR) status was evaluated by IHC staining. ER, PR, and HER2 positivity were assessed by counting the number of positive cells expressed by particle counting in a selected region of the same position of the carcinoma.

Immunohistochemical reaction

Estrogen and progesterone receptors, HER2, and Ki-67 markers in breast tissue were determined by IHC reactions. The sections were deparaffinized in xylene and rehydrated in graded alcohols and phosphate-buffered saline (PBS). The slides were incubated overnight with the primary antibody. After incubation with the primary antibody, the slides were washed with PBS and incubated with detection antibody for 30 minutes. A rabbit monoclonal antibody (dilution 1:1000) was used for ER testing and a mouse monoclonal antibody (dilution 1:1600) was used for PR testing. Diaminobenzidine was used as the chromogen, and the sections were then counter-stained with hematoxylin before being coverslipped. The results from the ER and PR assays were compared through image analysis. Positive staining was defined as nuclear staining in ≥1% of the tumor cells for ER and PR; HER2 was considered positive by the presence cytoplasmic staining.

Location of ROI in different slides

To locate an ROI from more than one histological or histopathological slide, we drew the shape of the stained tissue or marked points of the section onto a scanned copy (labeled as "marked slide" in Fig. 1 below) of a clean glass slide.

Fig. 1. The marked slide contains a hand-drawn mask of the landmarks on the parent slide. The use of this mask allows one to compare the same microscopic area from one slide to the same area from the same tissue block but that was stained with different stains on other slides.
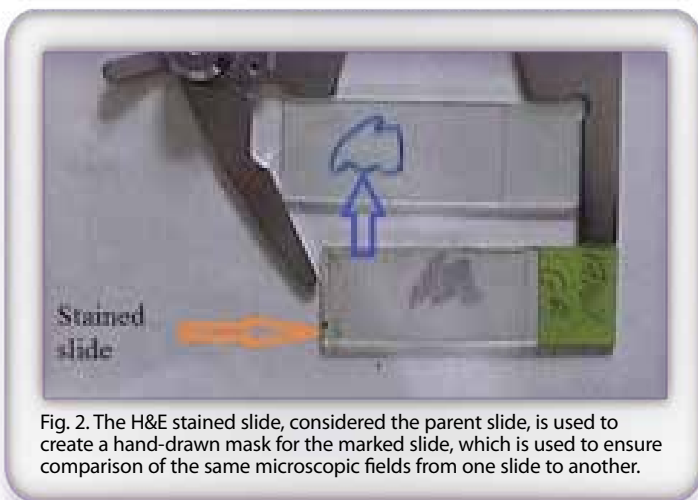

Fig. 2. The H&E stained slide, considered the parent slide, is used to create a hand-drawn mask for the marked slide, which is used to ensure comparison of the same microscopic fields from one slide to another.

We put the original stained slide defining the ROI next to the marked slide to match both regions (Fig. 2). In the marked slide, the drawn area becomes parent to the defined ROI that appears below it. Photographs were taken of an image for this ROI for the slide stained with H&E and stained immunohistochemically for expression of surface markers.

Repeat the same procedure for all slides you have and make photos.

Particle counting

Particle counting was done using ImageJ software. The previously photographed ROI was thresholded for both estrogen receptors (Fig. 3) and progesterone receptors (Fig. 4).

Fig. 3. Immunohistochemical staining of estrogen receptors (thresholded) in the studied breast cancer tissue. With the use of image analysis software (ImageJ, National Institutes of Health),[9] only the positively stained cells are visualized in this image, which facilitates quantitating the percent of positively stained cells.

Fig. 4. Immunohistochemical staining of progesterone receptors (thresholded) in a parallel section of the breast cancer tissue in Fig. 3. Only the positively stained cells are visualized in this image that was analyzed and quantitated using ImageJ software.

We used an 8-band threshold to determine the particle count in each related image using the displayed band for a gray scale display. As we navigated through different slides, it was critical that the image window be centered over the marked structure. The number of positively reactive cells (particle count) was then determined.

Results

Our method for localization of the ROI, which was the same for ER, PR, HER2, and Ki-67, is demonstrated from IHC reactive slides shown in Figs. 3A and 3B. The photomicrographs of those slides were processed using ImageJ image analysis. As measured by particle count from the image analysis, tumor cells that stained positive for estrogen receptors had 296 particles in nuclei of the tumor cells. Tumor cells that stained positive for progesterone receptors demonstrated 298 particles in nuclei of the tumor cells. The tumor cells stained negative for HER2 overexpression; the Ki-67 labelling was counted in 158 particles in nuclei of the tumor cells.

Discussion

Estrogen, progesterone, HER2, and Ki-67 were immunohistochemically assessed for a case of invasive breast carcinoma. The number of positive cells was measured by image analysis; the results were compared with those achieved with established antibody markers for estrogen receptors, progesterone receptors, and HER2 status. The results showed a high rate of concordance (similar number of positive cells in all reactions) between estrogen and progesterone but not for HER2, and a very low rate for Ki67 overexpression.

The immunohistochemical assessment of ER, PR, and HER2 is required for all patients with newly diagnosed breast cancer to determine the most effective treatment regimens.[10-12] A large proportion of these patients will have invasive breast carcinomas that are positive for hormonal receptors or HER2, making them eligible for treatment with hormone therapy or chemotherapy. These therapies bring significant benefits with respect to overall survival at 5 years and are associated with minimal morbidity compared with cytotoxic chemotherapy alone.[13,14] However, recent accounts show that erroneous ER and PR results can have devastating effects, including patients being denied appropriate therapy.[15]

The evaluation of ER and PR were comparable. The results obtained for estrogen and progesterone receptor status tests show whether or not one or both is causing the tumor to grow. It has been demonstrated that cancer that is hormone-sensitive is slightly slower growing and has a better chance of responding to hormone-suppression treatment than cancer that is hormone-receptor negative. The scores for hormone status are expressed as a number between 0 and 3. Scores depend on the percentage or number of cells out of 100 cells that test positive for hormone receptors in immunohistochemical tests, where 0% indicates no receptors and 100% represents all cells having receptors. If the score indicates hormone receptor-negative status, then the HER2 status of the tumor should also be evaluated to determine the most effective treatment. The most recognized tumor staging system is known as the TNM system. T represents tumor size, N represents lymph node status, and M represents metastasis.

Breast cancer is the most commonly occurring female cancer and the leading cause of cancer deaths worldwide. Breast cancer is a heterogeneous disease and it encompasses a variety of entities with distinct morphological appearances and clinical presentations. In recent years, it has become evident that this diversity is the result of genetic alterations.[16] The analysis of gene expression data has suggested that breast cancers can be divided into molecular subtypes that have distinct clinical features with markedly differing prognoses and clinical outcomes.[17] These subtypes consist of 2 ER-positive types (Luminal A and Luminal B) and 3 ER-negative types (HER2 expressing, basal-like, and normal breast-like).

Our method for concurrent localization of regions of interest in multiple slides that were subjected to different treatment and staining methods proved to be both efficient and easy to achieve.

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Educational Opportunities in 2015

JANUARY

28	**NSH Webinar 1:00 pm Eastern Time**
	Title: **Microwave Staining of Microorganisms**
	Speaker: Zoe Ann Durkin
	LabPulse Medical
	East Granby, CT
	Phone: (443) 535-4060 or register online at www.nsh.org

FEBRUARY

27	**NSH Webinar 1:00 pm Eastern Time**
	Title: **GHS (Global Harmonized System) Labeling**
	Speakers: Donna Chuddley
	Huntingdon Life Sciences
	East Millstone, NJ
	Chad McMahan
	Clemson University
	Clemson, SC
	Phone: (443) 535-4060 or register online at www.nsh.org
27-28	**Kentucky Society for Histotechnology**
	Title: **41st Kentucky State Symposium**
	Site: Galt House
	Louisville, KY
	Contact: Deborah Luttrell
	Phone: (502) 541-1271
	Email: debbieluttrell55@gmail.com

MARCH

6-7	**Indiana Society for Histotechnology Annual Symposium**
	Site: Fishers Banquet Center (meeting)
	Holiday Inn Express (rooms)
	Indianapolis, IN
	Contact: Debbie Wood
	Email: demwood@iupui.edu
20-22	**Texas Society for Histotechnology**
	Site: Dallas/Plano Marriott at Legacy Town Center
	Plano, TX
	Contact: Kathy Dwyer or Donna Willis
	Phone: (214) 980-4960 or (214) 725-6184
	Email: kdwyer3322@aol.com
	donna.willis@baylorhealth.edu
25	**NSH Webinar 1:00 pm Eastern Time**
	Title: **Histology and Histopathology Considerations for Animal Models**
	Speakers: Sue Knoblaugh, DVM, and Julie Randolph-Habecker, PhD
	Fred Hutchinson Cancer Research Center
	Seattle, WA
	Phone: (443) 535-4060 or register online at www.nsh.org

APRIL

9-11	**North Carolina Society of Histotechnology**
	Site: Doubletree Hotel at Research Triangle Park
	Research Triangle Park, NC
	Contact: Delorise Williams
	Phone: (919) 558-1252
	Email: dwilliams@thehamner.org
17-19	**Georgia Society for Histotechnology HISTOPALOOZA! 2015**
	Site: Legacy Lodge at Lake Lanier Islands
	Buford, GA
	Contact: Wanda K. Simons, HT(ASCP)
	Email: gshpresident@gmail.com
	Website: www.histosearch.com/gsh/
22	**NSH Webinar 1:00 pm Eastern Time**
	Title: IHC for Leukemia/Lymphomas
	Speaker: Madhu Menon, MD, PhD
	Henry Ford Hospital
	Detroit, MI
	Phone: (443) 535-4060 or register online at www.nsh.org
24-25	**Histology Society of Ohio**
	Site: Hilton Garden Inn
	Dayton/Beavercreek, OH
	Contact: Amy Aulthouse
	Phone: (419) 674-6087
	Email: a-aulthouse@onu.edu

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Educational Opportunities in 2015

MAY

1-2 **Michigan Society for Histotechnology**
    Site: Doubletree Hotel
            Bay City, MI
    Contact: Paula J. Canfield or Michelle Martin
    Email: pjcanfield@att.net or martmich@hotmail.com

6-8 **Tri-State Meeting (Iowa, Minnesota, Wisconsin)**
    Site: Concourse Hotel
            Madison, WI
    Contact: Dawn Schneider, Faith Mathews, Lois Rowe,
            Judith Stasko, or Jean Mitchell
    Phone: (715) 356-8174
    Email: dawn.schneider@ministryhealth.org
            mathewfm@ish.org
            rowe.lois@mayo.edu
            judithstasko@ars.usda.gov
            jmitchell@newcomersupply.com

14-15 **Illinois Society for Histotechnologists**
45th Annual State Meeting
    Site: Chicago Marriott Schaumburg
            Schaumburg, IL
    Contact: Lori Bellafiore
    Phone: (217) 202-3820
    Email: labellafiore@yahoo.com
    Website: http://illinoishistologysociety.org

27 **NSH Webinar 1:00 pm Eastern Time**
    Title: **Why Does H&E Staining Look Different Today?**
    Speaker: Ada Feldman
            Anatech Ltd
            Battlecreek, MI
    Phone: (443) 535-4060 or register online at www.nsh.org

JUNE

24 **NSH Webinar 1:00 pm Eastern Time**
    Title: **Ophthalmology for the Histotechnologist**
    Speaker: [need speaker info]
    Phone: (443) 535-4060 or register online at www.nsh.org

JULY

22 **NSH Webinar 1:00 pm Eastern Time**
    Title: **How to Integrate HistoQIP Into a Quality**
            Management Program
    Speaker: Janet Tunnicliffe
            Fraser Health Authority
            Surrey, British Columbia, Canada
    Phone: (443) 535-4060 or register online at www.nsh.org

AUGUST

26 **NSH Webinar 1:00 pm Eastern Time**
    Title: The Diagnostic Utility of IHC for Cytokeratins
    Speaker: Mohanpal Singh Dulai, MD
            Beaumont Health System
            Royal Oak, MI
    Phone: (443) 535-4060 or register online at www.nsh.org

28-Sep 2 **National Society for Histotechnology Symposium/Convention**
    Site: Washington, DC
    Contact: NSH Office
    Phone: (443) 535-4060
    Fax: (443) 535-4055
    Email: histo@nsh.org

SEPTEMBER

23 **NSH Webinar 1:00 pm Eastern Time**
    Title: **Basic In Situ Hybridization Theory and Background**
    Speaker: Traci DeGeer
            Ventana Medical Systems
            Tucson, AZ
    Phone: (443) 535-4060 or register online at www.nsh.org

OCTOBER

28 **NSH Webinar 1:00 pm Eastern Time**
    Title: **Histology and Special Stains of the Diagnostic Liver Biopsy**
    Speaker: Zhenhong Qu, MD, PhD
            William Beaumont School of Medicine
            Royal Oak, MI
    Phone: (443) 535-4060 or register online at www.nsh.org

NOVEMBER

18 **NSH Webinar 1:00 pm Eastern Time**
    Title: What Stains for What Tumor?
    Speakers: Sharon Lear and Alvin Martin, MD
            CPA Lab
            Louisville, KY
    Phone: (443) 535-4060 or register online at www.nsh.org

DECEMBER

16 **NSH Webinar 1:00 pm Eastern Time**
    Title: **Microsatellite Instability in Colorectal Cancer**
    Speaker: Beth Sheppard
            Ventana Medical Systems
            Tucson, AZ
    Phone: (443) 535-4060 or register online at www.nsh.org

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