Digital Imaging in Pathology: Whole-Slide Imaging and Beyond

Farzad Ghaznavi,[1] Andrew Evans,[2] Anant Madabhushi,[3] and Michael Feldman[4]

[1]Department of Pathology and Laboratory Medicine, University of Toronto, Toronto, Ontario M5S 2S2, Canada; email: farzad.ghaznavi@mail.utoronto.ca

[2]Laboratory Medicine Program, University Health Network, Toronto General Hospital, Toronto, Ontario M5G 2M9, Canada

[3]Department of Biomedical Engineering, Laboratory for Computational Imaging and Bioinformatics, Rutgers University, Piscataway, New Jersey 08854

[4]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; email: feldmanm@mail.med.upenn.edu

Annu. Rev. Pathol. Mech. Dis. 2013. 8:331–59

First published online as a Review in Advance on November 15, 2012

The *Annual Review of Pathology: Mechanisms of Disease* is online at pathol.annualreviews.org

This article's doi:
10.1146/annurev-pathol-011811-120902

Keywords

multispectral imaging, computer-assisted diagnostics, histocytometry, nonlinear dimensionality reduction

Abstract

Digital imaging in pathology has undergone an exponential period of growth and expansion catalyzed by changes in imaging hardware and gains in computational processing. Today, digitization of entire glass slides at near the optical resolution limits of light can occur in 60 s. Whole slides can be imaged in fluorescence or by use of multispectral imaging systems. Computational algorithms have been developed for cytometric analysis of cells and proteins in subcellular locations by use of multiplexed antibody staining protocols. Digital imaging is unlocking the potential to integrate primary image features into high-dimensional genomic assays by moving microscopic analysis into the digital age. This review highlights the emerging field of digital pathology and explores the methods and analytic approaches being developed for the application and use of these methods in clinical care and research settings.

DIGITAL PATHOLOGY AND WHOLE-SLIDE IMAGING SYSTEMS: AN OVERVIEW

Since the development of the first automated, high-resolution whole-slide imaging (WSI) system by Wetzel and Gilbertson in 1999 (described in Reference 1), interest in using WSI for different applications in pathology practice has steadily grown (1–3). All current WSI systems consist of illumination systems, microscope optical components, and a focusing system that precisely places an image on a camera. The final product, or virtual slide, can be assembled in various ways, depending on the particular scanner being used (tiling, line scanning, dual sensor scanning, dynamic focusing, or array scanning) (3). The result is a comprehensive digital rendering of an entire glass slide, visible at resolutions of less than 0.5 μm, that can be examined with interactive software on a computer screen (4). The viewing software closely emulates the performance characteristics of a light microscope in that the pathologist can freely navigate a digital image of a histological section over a complete range of standard magnifications (including oil immersion) and perform functions that have historically been carried out with a light microscope. WSI technology holds tremendous promise with respect to the digitization of pathology because it avoids many of the limitations imposed by earlier methods such as photomicroscopy (the capturing of selected representative images) and robotic microscopy (5). These approaches were limited by several factors, including suboptimal image quality, the inability of the viewing pathologist to see a high-resolution overview of the entire slide or to have control over its navigation, and the need for an extended amount of time to adequately review a slide (1, 3, 6–8).

Pathology, as with most medical specialties, is currently facing a growing demand to improve quality, patient safety, and diagnostic accuracy because there is an increasing emphasis on subspecialization. These factors, coupled with economic pressures to consolidate and centralize diagnostic services, are driving the development of systems that can optimize access to expert opinion and highly specialized pathology services. Digital pathology networks based on WSI systems provide a potential solution to all of these challenges and will undoubtedly play a critical role in this regard in the future (2). As with digital radiology, it is now believed that transformation to a soft-copy reading environment is possible for pathology as well. The emergence of more than 10 different WSI vendors over the past 5 years further indicates that pathology will eventually become a digital specialty. To date, however, the adoption of digital platforms by the pathology community as a whole has been slow, and the applications of WSI systems in pathology have been limited to education, research, and specific niches in clinical practice. Much work remains to be done before WSI technology for diagnostic purposes can be widely adopted (8–10). Arguably, the most important limiting factor is the perception among pathologists that WSI systems are inferior in terms of performance when compared with light microscopes. Given that pathologists have carried out their work with light microscopes for more than 100 years, WSI is considered a disruptive technology.

In this review, we focus primarily on the factors that currently facilitate or impede the adoption of WSI systems in pathology. We also review the limited but growing literature that describes the validation of WSI systems for diagnostic purposes and the use of this technology for actual patient care, multidisciplinary patient conferences (tumor boards), quality-assurance (QA) activities, and education.

ADOPTION OF DIGITAL PATHOLOGY SYSTEMS BY PATHOLOGISTS: FACILITATORS AND BARRIERS

One can easily identify advantages and disadvantages to switching from glass slides and light microscopes to WSI platforms. The adoption of digital technology by the pathology community has been slower than in radiology for

various reasons (11). Although pathology can learn lessons from radiology concerning the switch to digital reporting, there are key differences that prevent pathologists from simply reapplying the digital radiology template. These differences include our need for color images and the data-storage challenges that are created when large volumes of slides are completely digitized (12). In addition, radiology was able to eliminate films and all of the hazardous chemicals associated with producing them (13). The same cannot be said for pathology, wherein glass slides must still be produced and stored as well as scanned, which adds an extra step to the prediagnostic work flow.

Facilitators

Facilitators for the adoption of digital pathology can be considered in terms of cost savings, performance, work-flow efficiency, and access to pathology services in underresourced locations. The cost of replacing single-purpose microscopes with multipurpose computers and monitors can be shared among various departments as the number of WSI applications increases within and between institutions, which should stimulate growth in investment in this area over the next few years (7, 10, 14, 15). Digital pathology systems are also likely to be more ergonomically friendly than light microscopes, a factor that led a group in Kalmar, Sweden, to adopt the former technology to keep one of its members functioning as a pathologist (16). WSI systems can now be integrated with laboratory information systems to reduce errors related to specimen-patient mismatching. In terms of work-flow efficiency, WSI systems allow for more-streamlined navigation of slides at all magnifications by eliminating disruptions that can occur when a pathologist bumps a slide on the microscope stage (particularly when viewing a slide at high magnification). Computer-aided diagnostic tools will undoubtedly make pathologists more efficient and precise at quantifying histoprognostic factors such as mitotic figures.

Because relatively little is known about the cognitive factors that affect human performance in pathology practice, human-factor studies using digital pathology platforms have been performed. These studies have focused primarily on understanding the diagnostic pathways used by virtual slide readers in order to develop more usable interfaces for visualization of virtual slides, design more efficient digital reading environments, and improve the accuracy of digital slide interpretations (17, 18). It is more efficient to review virtual slides with residents in sign-out situations that involve more than one or two trainees. Residents can also have access to annotated online education modules, which is particularly advantageous for the independent study of rare or unusual cases without the need for a staff pathologist. Time and motion studies have convincingly shown that pathologists spend up to 15% of their professional time matching slides to paper requisitions and looking for glass slides for signing out, second opinion, tumor boards, research, and resident teaching. WSI systems with online digital archives have the potential to greatly reduce these inefficiencies and improve pathologist productivity (19, 20). Finally, WSI systems have tremendous potential to provide access to subspecialty pathology services for remote locations that have limited or no on-site pathology support. The digital approach obviates both the costs and time delays associated with shipping glass slides between centers and the risk of valuable slides being lost or damaged during transport. These potential benefits certainly make WSI systems an attractive alternative to traditional microscopy for a complete spectrum of activities in clinical pathology (2).

Barriers

Despite the numerous advantages outlined above, many technical and practical issues must be overcome before pathology can follow radiology's example in terms of going digital (11). These barriers can be considered in terms of cost and required infrastructure, image quality and speed of acquisition, data management,

standards, and regulatory approval, as well as pathologists' concerns with respect to inferior performance.

The cost of purchasing, implementing, and maintaining a WSI system can be prohibitive, depending on the scope of the digital pathology service under consideration, and especially for small pathology groups in nonacademic institutions or in situations where a compelling business case cannot be made. Apart from the cost of scanners (in excess of US$100,000–150,000 apiece), one must consider the cost associated with training of pathology staff and lab personnel, service contracts, technical support during the installation phase and ongoing use, digital slide storage and retrieval, and regulatory or licensure issues that may have to be addressed. There is also the possibility that labs will have to retrofitted with bar-code tracking systems, particularly if the WSI platform is to be integrated with a departmental laboratory information system.

Although robust, high-throughput slide scanners are now commercially available, their scanning speed and the acquisition of consistently well-focused digital slides are shortcomings (1, 4). Vendors in the digital pathology space are acutely aware of the need for fast WSI systems that provide image quality that matches or exceeds the visual experience obtained with light microscopy, and there has been progress in this area. Today's scanners can be loaded with 400 or more slides and can scan slides continuously; however, round-the-clock operation of multiple scanners is required to completely digitize the slide volumes of a typical academic pathology department (12), and rescanning is required for a variable percentage of slides. More than 1 min is still required to scan a typical 1.5 × 1.5 cm section at 20 × magnification. Most image-quality problems are focus related, and many can be traced back to the quality of the histologic section that was placed in the scanner. With current WSI technology, the quality of the slides to be scanned must be optimized in terms of uniform section thickness, placement of the section in the center of the slide such that it is completely covered by the coverslip, avoiding the creation of chatter artifact and tissue folds during microtomy, and avoiding the creation of air bubbles during coverslipping. All of these irregularities can adversely affect the focus and image quality of adjacent areas on the resulting virtual slide (**Figures 1** and **2**).

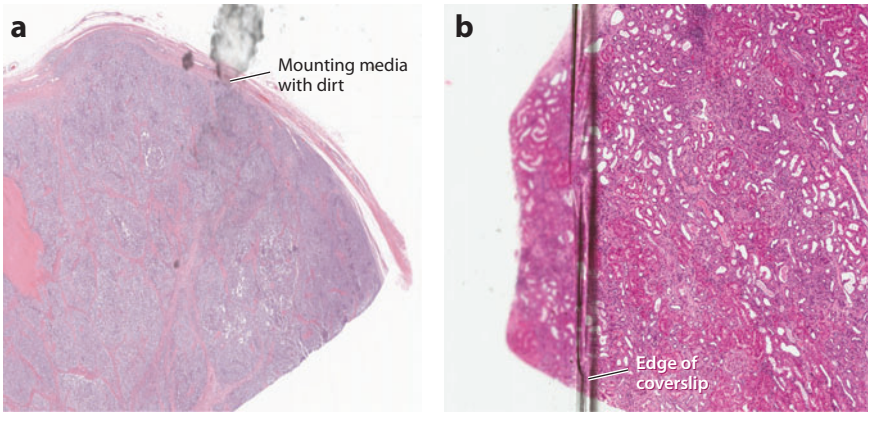

Figure 1

Representative examples showing the limitations imposed by suboptimal slide quality on the quality of images produced by whole-slide imaging devices. (*a*) Excess mounting media (with attached dirt) on the top of the coverslip adversely affect the focus of adjacent tissue. Dirty slides, such as this section from a seminoma, should be cleaned prior to scanning. (*b*) The coverslip on this kidney section has shifted, leaving the left edge of the section uncovered and out of focus. The coverslip should be reapplied and the slide rescanned.

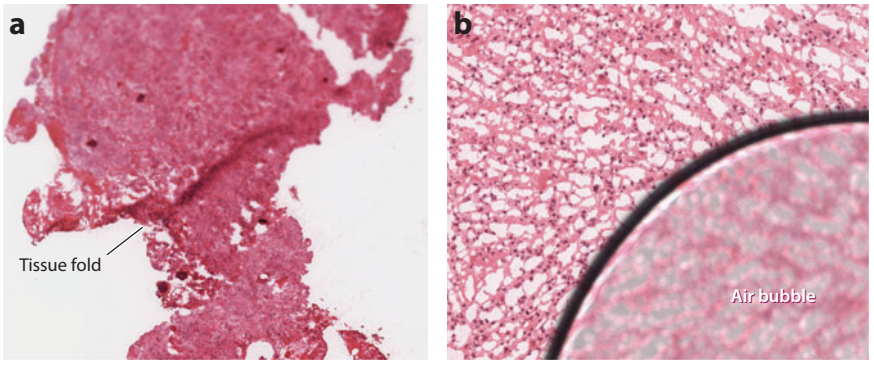

Figure 2

Representative examples showing the limitations on image quality imposed by suboptimal histologic processing of frozen sections read by whole-slide imaging. (*a*) A prominent tissue fold in this frozen section has adversely affected the focus of the top half of the image. Depending on the slide scanner being used, one must pay careful attention to avoiding the inclusion of tissue folds in the focus map that is generated prior to the scanning process. The alternative is to get a recut section without tissue folds. (*b*) Air bubbles likewise cause the affected area to be completely out of focus, rendering that portion of the slide unsuitable for assessment.

A recent study by Bautista & Yagi (21) investigated an automated method for detecting and avoiding these suboptimal areas during the focus-mapping stage of scanning; however, more work is required before this process can become successfully integrated into WSI scanners. Not all WSI systems can perform real-time multiplanar focusing to compensate for suboptimally focused areas. Although virtual focusing is now technically feasible, slides need to be scanned at multiple focal planes, which generates enormous image files (22, 23). For these reasons, current WSI systems are not suitable for reading cytology slides, wherein multiplanar focusing is frequently required to examine three-dimensional details of cells and cell clusters (24).

Development of an effective data-management system that can handle huge amounts of data (terabytes to petabytes), provide a streamlined image-retrieval process, and ensure security with currently available medical information systems remains an area of concern (9, 15, 25). Due to rapid technological advances in the area of digital processing and storage and the availability of effective compression algorithms, current difficulties related to the management of digital slide archives can be overcome. To illustrate this point, Huisman et al. (12) digitally archived all of the cases reported since November 2007 at University Medical Center in Utrecht, Netherlands. They undertook this project primarily to aid pathologists in their preparation for clinicopathological conferences. Three 120-slide scanners were used to continuously scan slides at 20 × magnification with a JPEG compression ratio of 70. More than 2,000 slides were scanned per week, on average, and the file sizes for individual slides ranged between 5 MB and 3.9 GB. This process generated roughly 40 TB of data annually, and by April 2009, the digital archive contained approximately 150,000 slides.

The increasing emphasis on quality and accuracy in pathology has created a growing need for pathology departments to follow best-practice standards and standard operating procedures, particularly when introducing new technologies such as WSI. The CLIA (Clinical Laboratory Improvements Act) requires all laboratory tests to be validated; however, it is not clear what part of examining a hematoxylin and eosin (H&E)-stained slide with a WSI system constitutes "the test" (5). The US Food

and Drug Administration (FDA) has not yet approved the use of WSI systems for H&E diagnosis, although the approval process has been initiated by several WSI vendors. Guidelines on monitor resolution and image quality and standards, such as the Digital Imaging and Communications in Medicine [DICOM, Supplements 122 (26) and 145 (27)], to create interoperability between WSI platforms, compression (JPEG 2000), and retention of digital slides used for diagnostic purposes are only beginning to appear (28). In addition, medicolegal and licensure guidelines governing the use of WSI systems for providing diagnostic services across jurisdictional boundaries have not yet been established. Because we are only just entering the digital era in pathology, it is not surprising that there is a paucity of guidelines. It is also not surprising that the pathology community is guarded about adopting a technology that lacks best-practice benchmarks.

Today's pathologists are under increasing pressure to handle large volumes of cases in a timely manner while providing an increasingly large amount of histoprognostic information in their consultation reports (especially in cancer cases). The stakes are high in that definitive surgical and medical treatments are based on the information provided by pathologists. In such an environment, pathologists are naturally wary about adopting digital systems that they think could both slow them down and increase the possibility of diagnostic error (11). Another key performance factor that remains to be evaluated is the possibility of visual fatigue (over and above that encountered with light microscopes) caused by signing out cases at a monitor for prolonged periods of time (29). Computerized simulations of a high-volume histology laboratory work flow carried out by McClintock et al. (30) provide a quantitative assessment of the feasibility for the full adoption of WSI in pathology practice. These authors' results, together with those from a study by Isaacs et al. (31), show that implementing WSI into high-volume pathology work flows has significant implications in terms of extra work, cost, and time.

OVERCOMING THE PERCEPTION OF INFERIOR PERFORMANCE: THE ROLE OF VALIDATION STUDIES

Even if most of the barriers are overcome, widespread adoption of WSI for primary diagnosis will not occur as long as pathologists believe that the performance of digital pathology systems is inferior to that of light microscopy (11, 32). The information required to surmount this important barrier can be obtained only by collecting objective data from well-designed validation studies that demonstrate the diagnostic equivalence (if not the superiority) of WSI systems to the light microscope. Such investigations will be necessary to gain regulatory approval from agencies such as the FDA, as well as to ensure pathologists' confidence in the diagnoses they make with these systems. In an effort to standardize the validation process, the College of American Pathologists has drafted recommendations on how to structure validation studies (33).

There is a relative paucity of peer-reviewed literature on validation studies for making primary diagnoses with WSI; however, new studies are steadily appearing in abstract form. In terms of published papers, Jukic et al. (34) investigated intrapathologist diagnostic discrepancy rates and diagnostic certainty between glass and digital slides by having three pathologists review 101 cases (900 slides in total from neoplastic and nonneoplastic cases) by both methods. They concluded that the use of WSI technology would not have adversely affected patient care or the diagnostic certainty of each pathologist.

A recent study by Mooney et al. (35) assessed the diagnostic accuracy and acceptability of virtual slides in dermatopathology. Ten pathologists and dermatopathologists were presented with a randomized series of 20 glass and virtual slides. These investigators found no significant differences in the diagnostic ability of the participants between the two modalities (0.85 for digital versus 0.81 for conventional microscopy; $p = 0.286$). Chargari et al. (36)

compared assessments of pathological features in a series of 816 prostate needle biopsy cores from 69 consecutive patients by using optical microscopy and digital means. Their results showed no significant difference in the percentage of biopsies in which cancer was detected (34.8% for conventional slides versus 33.4% for digital slides). Gilbertson et al. (4) demonstrated that WSI can be used for making routine diagnoses on genitourinary and dermatology cases. Although the three study pathologists raised concerns about areas on specific virtual slides that were suboptimally focused, this study reported complete agreement between WSI consensus diagnoses and gold standard diagnoses based on light microscopy. In a retrospective study by Fine et al. (37), five reviewers examined 30 diagnostically challenging prostate needle biopsies that required the use of immunohistochemical stains. The diagnostic performance of a WSI system was compared with that of light microscopy based on intra- and interobserver κ values, the time required to examine the cases, and information gathered from poststudy focus group discussions. Intraobserver agreements were reported as perfect for one reviewer, substantial for three reviewers, and moderate for the remaining reviewer. Although diagnostic agreement between each pathologist and the gold standard ranged from 0.52 to 0.73, agreement was in the excellent range ($\kappa = 0.817$) when comparing consensus WSI diagnoses with interpretation based on light microscopy.

A pilot study of 15 cases in Kyoto, Japan, conducted by Tsuchihashi et al. (38) suggested that rapid and accurate frozen-section diagnoses can be made by WSI. Fallon et al. (39) had two pathologists examine virtual slides created from 52 consecutive ovarian tumor frozen-section cases that covered benign, malignant, and borderline tumors. Although the reviewing pathologists did not have the full clinical information when they reviewed the virtual slides, they reported 96% concordance between WSI and the original light-microscopy diagnoses issued at the time of surgery. Interestingly, in some cases the WSI diagnoses were more accurate than those given at the time of surgery. Importantly, the discrepant cases were associated with well-known interobserver variability issues and were not considered to be a function of WSI.

Recently, Nielsen et al. (40) reported the diagnostic performance of virtual microscopy for routine histological diagnosis of skin tumors. Four pathologists who had limited experience in the use of virtual slides rendered diagnoses on 96 cases based on glass and digital sides (scanned at 20× magnification). They reported an overall diagnostic accuracy of 89.2% for virtual microscopy and 92.7% for light microscopy; the κ values were in the very good range for both intra- and interobserver agreement. Diagnostic discrepancies between WSI and light microscopy were attributed to the pathologists' lack of experience with the digital platform. These investigators concluded that it is feasible to use WSI systems to make diagnoses on the skin tumor types represented in the study.

A series of recent abstracts presented at the 2011 US and Canadian Academy of Pathology meeting has also provided encouraging validation data. Ramey at al. (41) reported complete diagnostic concordance of 91% between WSI and the original light-microscopy diagnoses issued for 72 consecutive frozen sections that were scanned at 20× magnification and subsequently reviewed by eight pathologists on both desktop and laptop computers. The overall κ value was 0.84. All the discrepancies were minor in nature and would have had no impact on intraoperative management. The type of frozen section had no influence on performance. In a companion abstract, the Ramey group (42) reported essentially identical results in terms of concordance when virtual slides of these frozen sections were reviewed on a high-resolution mobile device (an iPad). Finally, Reyes et al. (43) had three pathologists examine 103 breast needle biopsies by WSI (20× magnification scans) and light microscopy. These authors reported no disagreements with respect to distinguishing benign from malignant diseases.

All disagreements were associated with cases diagnosed as duct hyperplasia or atypical duct hyperplasia.

The validation studies summarized above indicate that WSI platforms can be used to make diagnoses that are as accurate as those made by light microscopy. The emerging theme from all of them is that when discrepancies between WSI and light-microscopy diagnoses arise, they tend to involve entities that are known to be plagued by interobserver variability. However, the problems with these studies are that they are limited to specific applications and that the results cannot be generalized to all areas of surgical pathology. Validation studies that cover the entire spectrum of cases and tissue types encountered in surgical pathology have not been performed, but will be required. A key issue, for which there are currently no guidelines, is how to design the ideal validation study. How many cases should there be? What about the mix of cases? How many pathologists should there be? Is the end point diagnostic concordance, feature recognition, or a combination of the two? What is an appropriate washout period between reviewing cases with WSI and light microscopy? How does one control for intra- and interobserver variability? In how many different centers do these studies need to be performed? These are only some of the issues that need to be considered, although it will probably be impossible to design a single perfect validation study (44).

VALIDATION OF WHOLE-SLIDE IMAGING FOR USE IN CONSULTATION MODELS

WSI technology is an obvious way to provide rapid consultation services to hospitals that lack on-site pathologists (45). The same would apply to solo pathologists in remote locations, who could benefit from expert consultations without having to incur courier costs, time delays, and the risks of losing or damaging slides if they are shipped to a referral center (37, 39). The number of studies evaluating WSI in consultation models is small; however, available data are encouraging. Rodriguez-Urrego et al. (46) recently published the results of an inter- and intraobserver agreement study in which four urologic pathologists compared WSI with light microscopy with respect to assigning Gleason scores and identifying other useful histoprognostic parameters in 50 challenging prostate biopsies in a consultation setting. Interobserver agreement in both methods was similar; the κ values ranged from 0.35 to 0.65 for all parameters. Intraobserver agreement was very good to excellent; the κ values for primary Gleason grade and Gleason score were >0.73. Tumor quantitation and perineural invasion also showed a high level of inter- and intraobserver concordance. These investigators concluded that WSI platforms would be sufficient for providing reliable consultation diagnoses on prostate biopsies.

A study by Wilbur et al. (47) looked at the feasibility of using WSI systems to provide consultation diagnoses for challenging cases from various anatomic sites. Fifty-three cases were assessed by two subspecialty pathologists, one using light microscopy and the other using WSI. They reported an overall concordance of 91% between WSI and light microscopy; neoplastic cases showed better concordance (93%) than did nonneoplastic cases (88%). Importantly, these investigators noted difficulties with navigation at high magnifications and in the interpretation of inflammatory or infectious lesions when using WSI.

USE OF WHOLE-SLIDE IMAGING FOR ACTUAL PATIENT CARE OUTSIDE OF VALIDATION STUDIES

Given the barriers described above, it should not be surprising that the literature describing the use of WSI systems for actual patient care, so-called off-label use, is sparse. Isolated abstracts have been presented that describe the use of WSI in a subspecialty consultation network in the United States (48) and for making primary diagnoses in the setting of a small group pathology practice in Kalmar, Sweden (16).

Starting in late 2006, Evans et al. (7) at University Health Network (UHN) in Toronto, Canada, have used WSI to make primary frozen-section diagnoses in the absence of an on-site pathologist. UHN is a three-site academic institution in downtown Toronto. The pathology department is consolidated in one site, leaving two sites without regular on-site pathologists for frozen-section coverage. One site is located approximately 1 mile from the consolidated department; it generates a low volume of frozen sections (typically fewer than 10 per week), the vast majority of which come from neurosurgery. WSI telepathology was implemented to deal with the inefficiencies associated with sending a single pathologist to cover frozen sections and the quality issues that may arise when a lone pathologist is faced with challenging cases in the absence of support from a colleague. This program is the first of its kind to use WSI for real-time diagnosis in patient care. On the basis of experience with more than 2,000 cases as of December 2011, single-block frozen sections are routinely reported with a total turnaround time of 14 to 16 min, with a deferral rate of $\leq$5% and a discrepancy rate of $\leq$2% (when comparing intraoperative WSI diagnoses with the diagnoses provided by light microscopy at final sign-out). This program has leveraged the ability of WSI to enable real-time consultation on all cases in which the primary pathologist is considering deferring a frozen-section diagnosis. Pathologists have used WSI to make reliable interpretations of smears (or squash preps), which are often important in intraoperative neuropathology. It has been their experience that 20 $\times$ magnification scans are sufficient for assessing both frozen sections and smears, and image quality is not a problem if well-prepared slides are placed in the scanner (**Figure 3**). **Figure 4** shows an approach to scanning intraoperative smear slides that minimizes the area of the smear that needs to be scanned and optimizes the focus of cells at the diagnostic (thinnest) end of the smear.

The WSI telepathology program at UHN has recently been expanded to provide primary frozen-section support, without incident, to a hospital 400 miles north of Toronto when there is no on-site pathologist (A. Al Habeeb, A. Evans, S. Serra & R. Vajpeyi, unpublished observations). This system also facilitates the introduction of quality measures, such as rapid consultation between colleagues, when there is an on-site pathologist (3, 7).

MULTIDISCIPLINARY PATIENT CONFERENCES

Multidisciplinary conferences, or tumor boards, play a central role in decision making for quality cancer care. Although tumor boards may seem an obvious application for WSI technology, there is, once again, a paucity of literature on the subject. Spinosa (49) provided a comprehensive overview of a pilot project investigating the effectiveness of using WSI for tumor boards at Scripps Memorial Hospital La Jolla in California. This study demonstrates benefits such as increased efficiency for pathologists when preparing cases for presentation, improved quality in terms of information that is presented to clinical colleagues, and increased satisfaction on the part of all who attend these meetings.

QUALITY ASSURANCE

All medical specialties face increasing pressure to improve quality and patient safety. In pathology, the public attitude has shifted toward expectations of faster and more patient-centric subspecialty service that minimizes diagnostic errors (50–52). QA plays a central role in this process, and indeed, a growing number of institutions are adopting QA policies whereby a given percentage of cases must be independently reviewed by a second pathologist before sign-out (1, 50). Several studies, using different approaches, have investigated the incidence and characteristics of discrepancies between original and second reviews when both are performed using only glass slides. Manion et al. (53) reviewed 5,629 surgical pathology cases at the University of Iowa Hospitals and Clinics

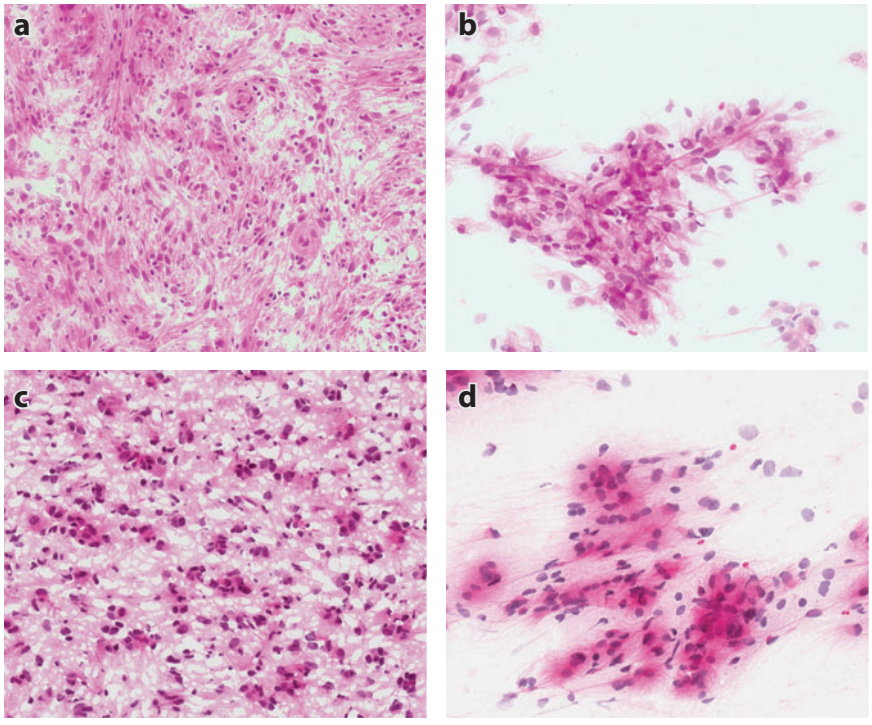

Figure 3

Representative examples of the image quality that can be obtained from 20× magnification scans of well-prepared frozen sections and intraoperative smears. (*a*,*b*) A meningioma frozen section and a paired smear, respectively. (*c*,*d*) A low-grade astrocytoma frozen section and a paired smear, respectively.

and found 132 (2.3%) major disagreements, of which 68 resulted in changes in clinical management. In a nonconcurrent cohort study by Raab et al. (54), pathologists reviewed glass slides from a total of 7,824 in-house cases, of which 7,444 were selected using a targeted 5% random review process and 380 were chosen for focused review. The total number of discrepancies detected by random review was 222, of which 27 (12%) were considered major ($p < 0.001$). Through the use of focused review, 12 of 62 (19.4%) discrepant cases were considered major ($p < 0.001$). Published results based on reviews of outside material report discrepancy rates ranging from 1.4 to 11.3%; almost 60% of major discrepancies resulted in changes in clinical management (55).

QA is an area in which WSI technology can play a critical enabling role. Digital pathology networks can avoid costs and potential difficulties associated with transporting glass slides between facilities. They can also be set up in such a way that potential second-reviewer bias, in favor of or against the original diagnosis, can be minimized (1, 56).

One of the first pilot studies demonstrating the potential of using WSI for QA purposes came from the University of Pittsburgh Medical Center in 2006. In this study by Ho et al. (1), 24 full genitourinary pathology cases with significant diagnostic complexity (comprising 47 separate parts and 391 slides) were rereviewed by three pathologists using WSI and traditional light microscopy. All of the reviewers had prior experience in reading digital slides. Four clinically insignificant discrepancies were found, two of which were based on the WSI review and two on light

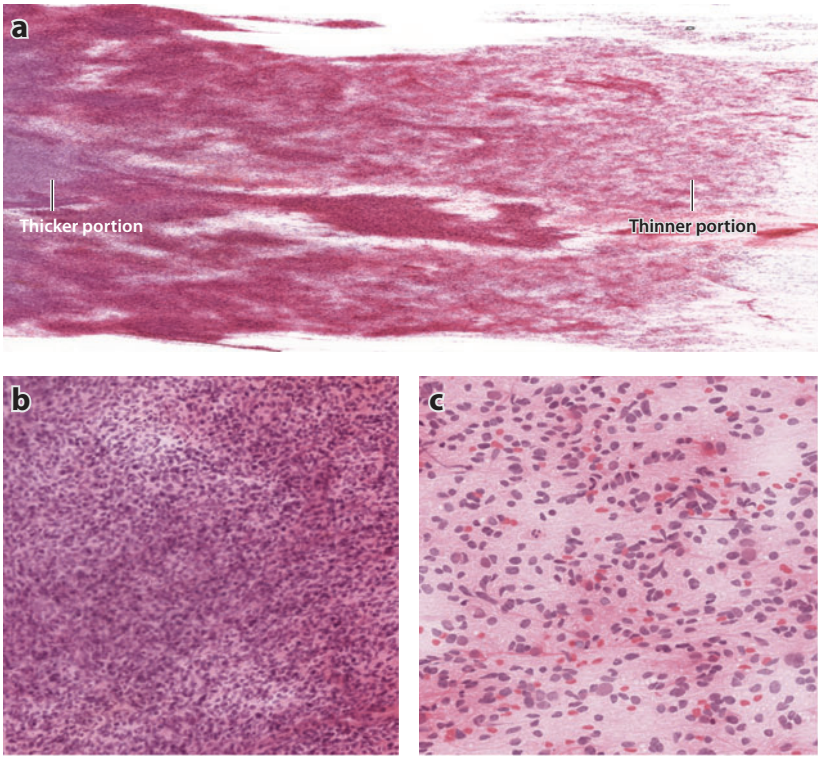

Figure 4

An approach to handling intraoperative smears (squash preps) by whole-slide imaging, as illustrated with a high-grade astrocytoma. (*a*) An overview of the entire smear. The area adjacent to the location where the tissue was placed on the slide prior smearing is thick (*left*), whereas the distal portion of the smear is much thinner (*right*). (*b*) Including the thick portion on the smear in the area to be scanned creates a much larger digital slide file, prolongs the scanning time, and produces a suboptimally focused image (in the absence of the ability to perform real-time multiplanar focusing). (*c*) Limiting the scanned area to the thinner, distal portion of the smear reduces scanning time and maximizes the image quality in terms of cytological detail at the diagnostic end of the slide.

microscopy. No significant concerns were raised by the study pathologists with respect to image quality, and all of them agreed that WSI is a viable foundation on which to build a QA program in a multisite health care facility.

In a recently published review, Graham et al. (56) described a WSI-enabled QA program at the University of Arizona. This system is used to provide same-day QA reviews between two hospitals located 6 miles apart in Tucson. One of these sites is a high-volume center staffed by several pathologists, whereas the other has a considerably lower volume of work and is staffed by a single part-time pathologist. During daily QA conferences, all new cancer cases and other difficult cases encountered at the low-volume site are scanned and rereviewed by staff pathologists and pathology residents at the larger site. This study has nicely shown that the vast majority (>95%) of QA work between two or more centers can be performed using WSI without having to transport glass slides. On the basis of QA of 329 cases between March 2006 and September 2008, there was complete diagnostic agreement in 91.8% of them. Minor discrepancies that would have had no impact on patient care were noted in 3% of the cases, and major discrepancies that

would have resulted in different treatment were found in 1.5%. In 1.8% of the cases, the QA diagnoses were deferred pending review of the original glass slides.

Another study conducted at the University of Arizona (51) described the performance of a WSI-based same-day second-opinion service for 154 newly diagnosed breast cancers in a four-site institution. As with the earlier report from Graham et al. (56), the vast majority (>95%) of QA work could be accomplished using digital slides. The breast cancer QA program identified a small number (1.9%) of minor discrepancies. Major discrepancies that would have resulted in different treatment were found in 2.3% of rereviewed cases, 1.9% of cases were deferred for further immunohistochemical staining, and 1.3% were deferred pending review of the original glass slides.

CLINICAL EDUCATION AND COMPETENCY ASSESSMENT IN PATHOLOGY

Because of efforts to satisfy the demand for high-quality pathology services, there is a growing need for systems that can efficiently assess the diagnostic proficiency of pathology trainees and staff pathologists (57, 58–60). WSI is a logical platform on which to build such education and proficiency-testing programs (61, 62). Importantly, WSI platforms ensure that exactly the same slides can be simultaneously reviewed by all participants. WSI provides an effective means of annotating images for instructional purposes and creating digital slide archives that can easily be coupled with the relevant clinical and/or radiological information. These approaches improve teaching efficiency and increase pedagogic versatility (14). The experience at the University of Oklahoma Health Sciences Center (63) shows that integrating WSI into their online pathology education program has enabled content such as annotated digital slides and an online WSI atlas. van den Tweel & Bosman (64) recently reported on the benefits of incorporating virtual slides into a system known as EUROPALS (European Pathology Assessment and Learning System) to assess the diagnostic skills and theoretical knowledge of pathology trainees across Europe. Despite technical challenges from both server and user sides, the use of virtual slides provided greater flexibility than did selected static images.

Several institutions, including the University of Iowa in the United States (14), the University of Basel in Switzerland, and the University of Saarland in Germany, have successfully implemented digital technology in their undergraduate medical curricula (65, 66). Emerging data suggest that today's medical trainees prefer teaching modules based on WSI systems to those based on light microscopy and glass slides (65). To assess the effectiveness of virtual microscopy for teaching purposes, Collier et al. (67) interviewed 12 teaching assistants from an undergraduate human anatomy course. They found that the majority of interviewees cited ease of use, universal access to teaching material, and increased student collaboration as advantages of the new technology. Fonyad et al. (59) summarized their 4-year experience of using digitalized histology labs in graduate student education at Semmelweis University in Hungary. Between 2007 and 2009, their digital histology lab served 928 students with a virtual slide set comprising predominantly H&E slides scanned at 20× magnification. These authors reported high user satisfaction with the WSI approach. The University of Pittsburgh has successfully implemented a Web-based digital teaching model for genitourinary pathology (60). Bruch et al. (58) have similarly developed a WSI-based tool for assessing the competency of pathology residents at the University of Iowa. This program allows them to follow an individual resident's progress throughout the course of his or her training. These authors concluded that their model can be applied across multiple pathology residency programs. A survey conducted at the University of Queensland School of Dentistry in Australia (68) indicated that undergraduate students were reluctant to use

traditional light microscopes and were heavily in favor of learning through virtual microscopy. A majority of students (>88%) felt that the virtual slide method increased their engagement with course content. Finally, two Web-based virtual microscopy applications in breast pathology and Gleason grading of prostate biopsies indicated that WSI systems provide a robust platform for educational purposes (69, 70).

CYTOMETRIC ANALYSIS OF PATHOLOGY MATERIALS: BACKGROUND

For decades, pathologists have been using immunohistochemistry (IHC) as an adjunctive tool to evaluate protein-expression patterns in tissue. This process assists in diagnosis by finding protein-expression patterns that correlate with the type of tumor (e.g., carcinoma, sarcoma, lymphoma, or melanoma) or, more specifically, the site of a primary tumor when an occult metastasis is identified. Although few single proteins define a site of origin, combinations of stains often allow pathologists to predict the probable site of a tumor's origin. More recently, immunohistochemical stains have been used to quantitate biomarkers to assist in therapeutic drug selection. Perhaps the best-known example of this application is in breast cancer management. Expression of hormone receptors for estrogen (ER) and progesterone (PR) are semiquantitatively measured using IHC. The resulting Allrad score can then be used to select hormone targeted therapy in tumors that are ER positive. In addition, overexpression of the epidermal growth factor receptor (EGFR)-family protein HER-2/neu is also measured semiquantitatively to evaluate for protein overexpression (usually associated with gene amplification) for a selection of patients who may respond to antibody therapy to the *Her-2* gene product, such as trastuzumab.

Although such visual, analog, pathologist-driven scoring systems have been used for decades to evaluate protein expression, recent advances in WSI, multispectral imaging, and immunofluorescence microscopy, combined with automated image-analysis tools, have begun to allow pathologists to consider new paradigms for automated scoring of IHC studies. Importantly, these new technologies allow pathologists to consider adopting new staining protocols, including multiplexed antibody studies, which are very difficult, if not impossible, to accurately quantitate using historical analog-driven approaches. These new digital methodologies also allow for the broader adoption of immunofluorescence, rather than IHC, as a primary diagnostic tool. Immunofluorescence has many advantages over IHC, including the ability to develop high-order multiplexing. Immunofluorescence is also linearly related to the amount of antibody bound to the tissue, which renders it more suitable for reproducible quantitative studies. A significant limitation of immunofluorescence studies is the need for the end-user pathologist to spend time in dark rooms at a fluorescence microscope. New digital imaging platforms obviate the need for the pathologist to drive a fluorescence scope and allow imaging technicians to perform the primary image capture and automated analysis; the pathologist reviews and integrates the information from these systems with the morphology to create a comprehensive disease report. If this paradigm sounds familiar, it should. It is the exact paradigm used in the practice of hematopathology, in which a technician performs multiplexed immunofluorescent stains (four to six stains at a time) by using a flow cytometer. (It is a rare hematopathologist who has time to run the flow cytometer in today's busy clinical environment.) The technician returns the resulting flow scatter plots to the hematopathologist for interpretation and integration into the hemepath report. If data need reanalysis, the hematopathologist directs the technician how to perform the reanalysis. This is the future of cytometric analysis of tissue sections, which will be enabled with the advent of the technologies discussed in the next section, namely multispectral histocytometric

image analysis and immunofluorescence histocytometric image analysis.

MULTISPECTRAL IMAGING: BACKGROUND

Spectral microscopic imaging refers to the capture of spectrally resolved information at each pixel in an image—in essence, spatial spectrophotometry. The number of wavelengths of the information captured distinguishes multispectral (10–30 bands of data) from hyperspectral (hundreds to thousands of bands of data) imaging systems. Spectral microscopy, therefore, is a specialized form of digital microscopy designed to capture spatially resolved spectral information (using bright field, fluorescence, or even a combination of the two). Although it is feasible to capture a multispectral image of a whole slide, this is not routinely done because only a few dozen fields of information are required to create a statistically meaningful sample of a tissue slide. The resulting spectral information captured by a spectral imaging system is a data cube that comprises *x* and *y* coordinates of information from the charge-coupled device (CCD) sensor; each *z* plane in the data cube provides information about intensity at each pixel as a function of wavelength (*z* axis of the data cube). **Figure 5** illustrates the creation of a spectral data stack.

Whole-slide digital imaging systems currently on the market use red-green-blue (RGB)-based imaging methods. Spectral imaging systems offer advantages over RGB systems, including the ability to analyze pathology slides stained with multiple antibodies (in either bright-field or fluorescence mode). Spectral imaging systems also permit the use of fluorescence imaging by overcoming autofluorescence, which is very commonly observed in formalin-fixed paraffin-embedded tissue. Autofluorescence removal is accomplished by directly measuring autofluorescence spectrally and then unmixing (by using a curve-fitting algorithm to separate the spectral fluorescence curve attributed to autofluorescence from the spectral curve associated with a specific fluorophore). Although both the autofluorescence and the fluorophore may appear green, they are spectrally different. Through the removal of autofluorescence, spectral imaging allows pathologists or researchers to use single or multiplexed fluorescence imaging methodologies in routine surgical pathology. Finally, spectral imaging enables computer analysis of routine stains (H&E- or Papanicolaou-stained samples) in order to develop automated machine classification systems to predict disease types or outcomes (71–73). Like fluorescence imaging, the application of multispectral imaging in bright field–based IHC application allows for the use of multiplexed immunohistochemical stains that are in spatially overlapping cellular compartments (74–77). The ability of whole slide–based RGB imaging systems to resolve more than three colors of information is, in practice, impossible. RGB imaging systems are not able to adequately deconvolve or separate the chromogens for analysis. In contrast, spectral imaging systems can resolve three of more chromogens. **Figure 6** shows a multispectral data stack of breast carcinoma stained with DAPI (4′,6-diamidino-2-phenylindole) and EGFR, comparing (*a*) conventional fluorescence imaging with monochrome band passes without autofluorescence removal with (*b*) the same field of view imaged using a spectral system with autofluorescence removal. Multiplexing more than two antibody labels is not done in routine pathology practice at present; however, the ability to simultaneously label more than two proteins in a single slide will allow for the development of cellularly resolved information about pathways (e.g., an antibody to phosphorylated ERK, a member of the mitogen-activated protein kinase signaling pathway), cell fate (e.g., a second antibody to Ki-67) and cell types (e.g., a third antibody to cytokeratin to find tumor cells in a field of view versus nontumor stromal areas). Roysam and colleagues (78, 79) have developed a system for the analysis of cytometrically resolved multiplexed IHC or immunofluorescence. This slide-based system is

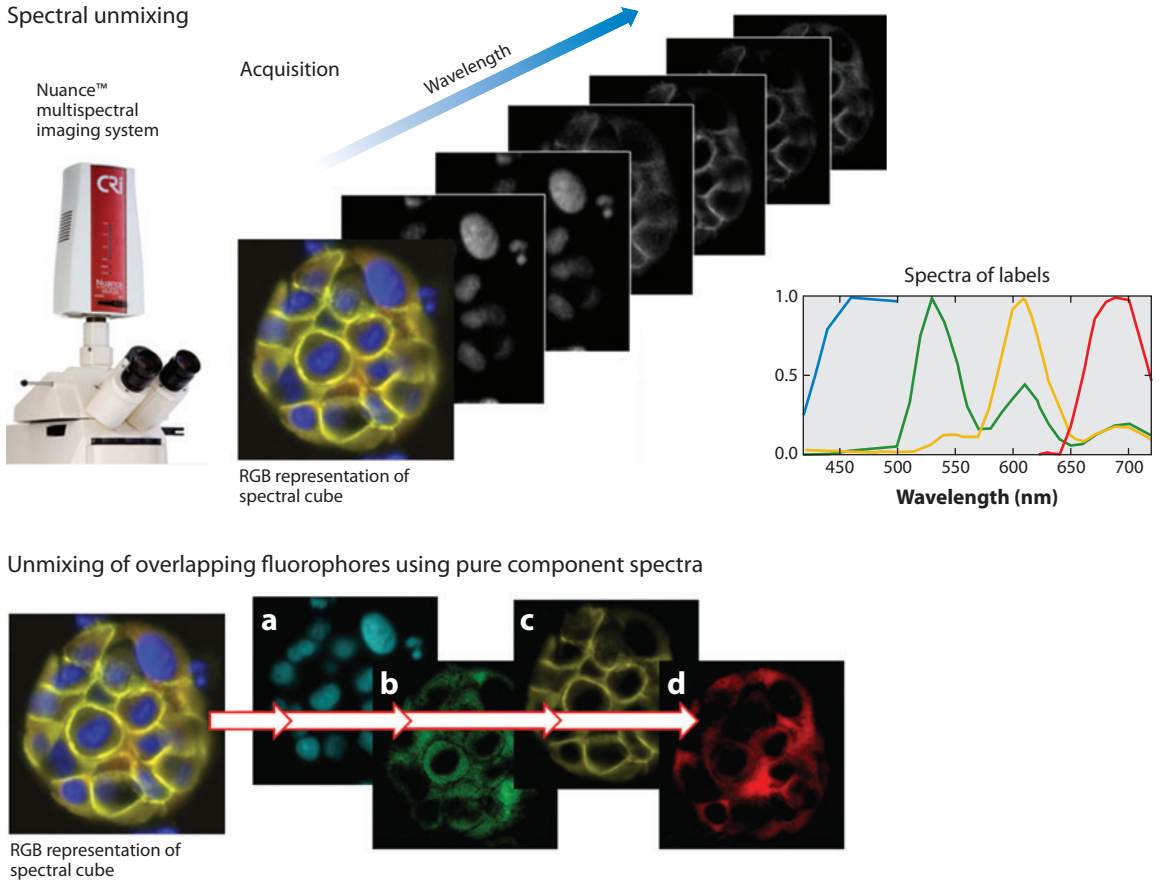

Figure 5

A multispectral imaging system and the resulting spatially aligned image stack are illustrated for a carcinoma stained with DAPI (4′,6-diamidino-2-phenylindole) and three fluorescently labeled antibodies. In this case, a liquid-crystal tunable filter multispectral imaging system from CRI, Inc. (Nuance™) was used to capture the data from 420 to 720 nm. The spectral profiles of the four fluorochromes are shown: (*a*) DAPI, (*b*) Alexa 488, (*c*) Alexa 594, and (*d*) Alexa 660. With a known spectral library of the pure fluorochromes, linear unmixing allows the individual fluorochromes to be separated from the complex mixture in the original starting image, shown as a red-green-blue (RGB) image.

analogous to flow cytometry and is aptly named quantitative histocytometry. Applications of quantitative histocytometry are explored further below. However, the implications of quantitatively measuring cell-signaling pathways and linking them to cell fate (proliferation, apoptosis, or autophagy) will be significant as pathologists enter the era of personalized diagnostics and the development of companion diagnostics for pharmacologic agents that target signaling pathways, of which there are hundreds in various stages of clinical trials.

MULTISPECTRAL HARDWARE

Spectral imaging requires specialized imaging hardware that is different from WSI systems. A means of generating spectrally encoded information is required. There are multiple methods for creating the spectral data, including liquid-crystal tunable filters (LCTFs) and tunable light sources, acousto-optical methods, diffraction gradients, and fixed-filter methods (80). Three of these systems are commercially available: the LCTF (CRI, Inc.; see **http://www.cri-inc.com**),

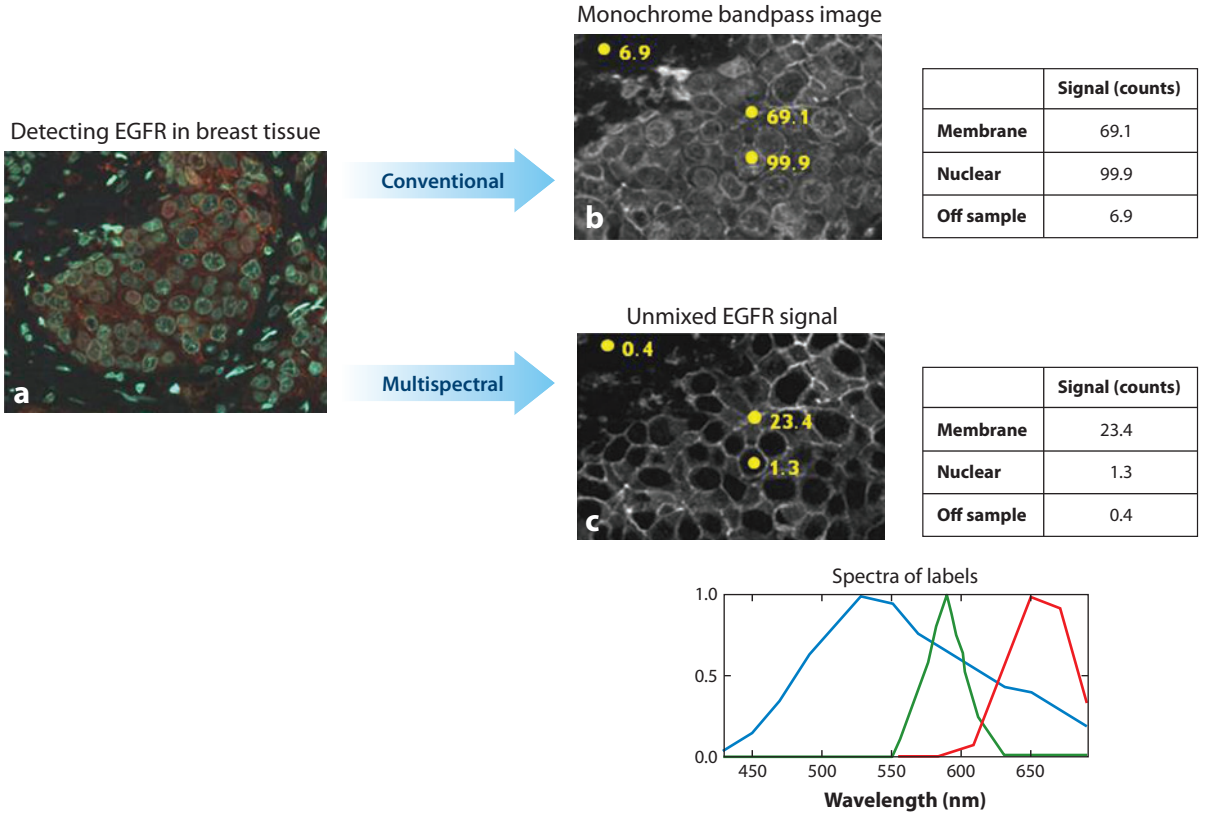

Figure 6

A breast carcinoma case stained for DAPI (4′,6-diamidino-2-phenylindole), epidermal growth factor receptor (EGFR) (Alexa 594), and estrogen receptor (Alexa 547) is (*a*) shown as a red-green-blue image and then analyzed (*b*) by conventional monochrome band-pass filters without autofluorescence removal or (*c*) after autofluorescence is removed following multispectral image acquisition. The removal of autofluorescence, followed by unmixing of the two antibodies, allows for precise measurement of the EGFR membrane signal.

the prism-based method (Lightform; see **http://www.lightforminc.com/**), and the fixed- or diffraction methods filter (Zeiss; see **http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/index.html**).

In general, spectral imaging systems allow for the capture of spectrally resolved information across the visible range and into the near-IR bands (1-nm band passes between 420 nm and 900 nm). These systems allow transmission of saturated colors within narrow spectral bands that can be electronically and randomly changed (or tuned) to any wavelength. Spectral imaging systems typically couple the spectral imaging hardware (LCTF or a prism or diffraction system) with coupling optics and a cooled, scientific-grade monochrome CCD into an integrated imaging platform. Software coordinates the image-acquisition process. These systems are capable of fluorescence-based analysis or bright field–based projects for chromogen-based assays. Image acquisition is accomplished via a software interface that can be automated. The resulting data cube (*x* and *y* coordinate position on a CCD sensor; the *z* axis represents intensity data at different wavelengths) is then stored for further analysis.

SOFTWARE (ANALYSIS OF SPECTRAL IMAGES)

Images acquired using a spectral imaging system can be either further analyzed by unmixing

(multianalyte assays or for object classification systems) or used in classification projects in which the spectral signature of different object classes is used to discriminate cells or tissue on the basis of routine sample preparation (H&E- or Papanicolaou-stained materials). The use of spectral images to classify disease processes has shown promise in some disease states. El Diery and colleagues (81) have used spectral spectral imaging to distinguish benign from neoplastic melanocytic lesions, with good success. In cytopathology, Rimm and colleagues (71–73) have used multispectral imaging to classify bladder cytology samples for the identification of high-grade dysplastic cells and thyroid cytology samples for thyroid neoplasia, with very good success. Classification of these different disease states may occur according to simple differences in the spectral signatures of the different disease states, or it may require more complex analysis of the spectral patterns inherent within different disease classes (described below in the section titled Image Analysis).

In addition to the application of multispectral imaging for disease classification, a more common use of spectral imaging is in the analysis of tissue stained with one or more antibodies, either in fluorescence or bright-field imaging applications. Analyses of multianalyte problems begin by unmixing or spectrally separating the individual stains into their individual spectra. Unmixing begins with pure spectra of the individual stains, which are obtained by staining the tissue with one antibody or counterstain alone before applying them in combination. The individual spectra of each stain can then be directly measured and used to build a spectral library. Through the use of the individual pure spectra, unmixing is based on a least-squares curve fitting for linear unmixing (77). The resulting unmixed spectra then show the intensity contribution of each stain at every pixel in the image, thereby allowing for additional image analysis and classification schemas (described in detail below). In addition to linear unmixing, some applications are best performed using alternative algorithms, including nonlinear unmixing approaches such as spectral waveform cross-correlation analysis (81). These alternate spectral unmixing algorithms are especially valuable when linear unmixing approaches fail [i.e., when stains do not obey Beer's law (82)].

STAINING LIMITATIONS PLUS AUTOFLUORESCENCE

The use of fluorescent dyes in formalin-fixed paraffin-embedded tissues has not been widely adopted in clinical practice. It is mostly seen in research settings. A major limitation of using fluorescent dyes has been the challenge of autofluorescence. Different tissues from different people have variable amounts of autofluorescence. Autofluorescence may be so intense that it overwhelms the signal from antibodies labeled either directly or indirectly to fluorochromes. Removal of autofluorescence can be accomplished by independently measuring the autofluorescence of the tissue and subtracting it from the combined signal of autofluorescence plus specific fluorochrome(s). Use of a least-squares fit algorithm to remove autofluorescence leads to between 100- and 1,000-fold suppression of autofluorescence, allowing visualization of underlying fluorescently labeled antibodies (**Figure 6**). Other approaches use nonspectral imaging approaches and provide similar amounts of autofluorescence suppression (83). The additional advantages of fluorescence compared with standard chromogenic IHC include a more linear relationship between antibody binding and fluorescence signal intensity, which makes quantitation and calibration much more uniform and reproducible (84, 85). Fluorescence also allows for the development of multiplexed antibody studies in tissues. One can use multiple antibodies with chromogenic IHC when the dyes do not spatially overlap. However, when two or more antibody stains overlap in the same spatial compartment (i.e., there are two nuclear signals), it can be challenging to separate the individual signals when they are chromogenic. Whereas two stains can be separated from each other (using color deconvolution for standard RGB-based systems, or using spectral

unmixing), the addition of a third or fourth antibody in the same spatial cellular compartment causes these separation techniques to fail because color deconvolution uses only three data channels (RGB channels) to unmix multiple colors. Also, the aggregation of multiple chromogens causes the role of chromogens to change from light absorber to stronger light scatterer, and at very strong stain intensities, the scattering properties of the chromogens prevent light from passing through the aggregated chromogens.

HISTOCYTOMETRIC ANALYSIS OF MULTISPECTRAL IMAGES

Antibody staining to reveal specific molecular biomarkers is increasingly being used to improve cancer diagnosis and classification, establish prognosis, and determine therapy. Although molecular biomarkers play an increasingly large role in this process, the scoring of stained specimens (immunohistochemically or immunofluorescently) remains largely visually subjective: Cells are scored as positive or negative or are graded for degree of antigen staining, the percentage of positive cells is estimated, and overall scores are binned or scaled using semiquantitative approaches. This process requires considerable expertise and is susceptible to interobserver variability, despite standardization efforts (86–88). The use of semiquantitative scoring (e.g., 0, 1+, 2+, 3+ staining) and H-scores acknowledges the inherent imprecision and subjectivity involved.

Computer-automated methods to quantify antigen expression in tissue images have been developed (89–92); these methods offer objectivity, reproducibility, and quantification on a continuous scale. Most operate by measuring the number of pixels stained for one or more antigens and by quantifying the colocalization of stains. These methods can quantify at the level of individual pixels, groups of pixels, or image regions. Although such pixel-based approaches offer improvements in quantitation and reliability over manual scoring methods, they are not performed on a cytometric basis (i.e., individual cell analysis). The HistoRx platform (see **http://www.historx.com/launch/index.html**) is perhaps the most advanced pixel-level automated image-analysis system available today; multiple studies have shown the advantage of automated quantitation using this platform compared with the manual scoring of pathology samples (84, 85, 89, 93, 94). Cells, rather than pixels, are the fundamental units in which many biological processes occur. Sufficiently reliable automated methods to segment (delineate) individual cells, identify subcellular compartments within cells, and quantify biomarkers within the subcellular regions have only recently been developed. Roysam and colleagues (79) have developed an open source–based cytometric analysis system (Farsight; see **http://farsight-toolkit.org**) for the analysis of cells in surgical pathology samples. Using tissues stained with hematoxylin and DAB (diaminobenzidine) or DAPI and fluorochromes, Farsight uses the nuclear channel (either DAPI or hematoxylin) to perform a nuclear segmentation process by converting the image into a binary map, then finding and refining the center of nuclei to segment the nuclear contours. Following nuclear segmentation, a cell-membrane marker in another spectral channel is used to define the cell membrane (e.g., E-cadherin is used for breast carcinoma). The resulting nuclear and membrane boundaries are then utilized to define the cytoplasmic area by use of an adaptive algorithm that switches between different cell-based models on a cell-by-cell basis. The resulting cellular segmented maps with associated subcellular localization can then be used to associate additional staining information from additional analyte channels from the multispectral assay on a cell-by-cell basis with subcellular localization to the nucleus, cytoplasm or cell membrane. The resulting output data set resembles a list-mode data file from a flow cytometer in that each row of data represents a cell and each column a marker of interest within a subcellular compartment of the individual cell (**Figure 7**).

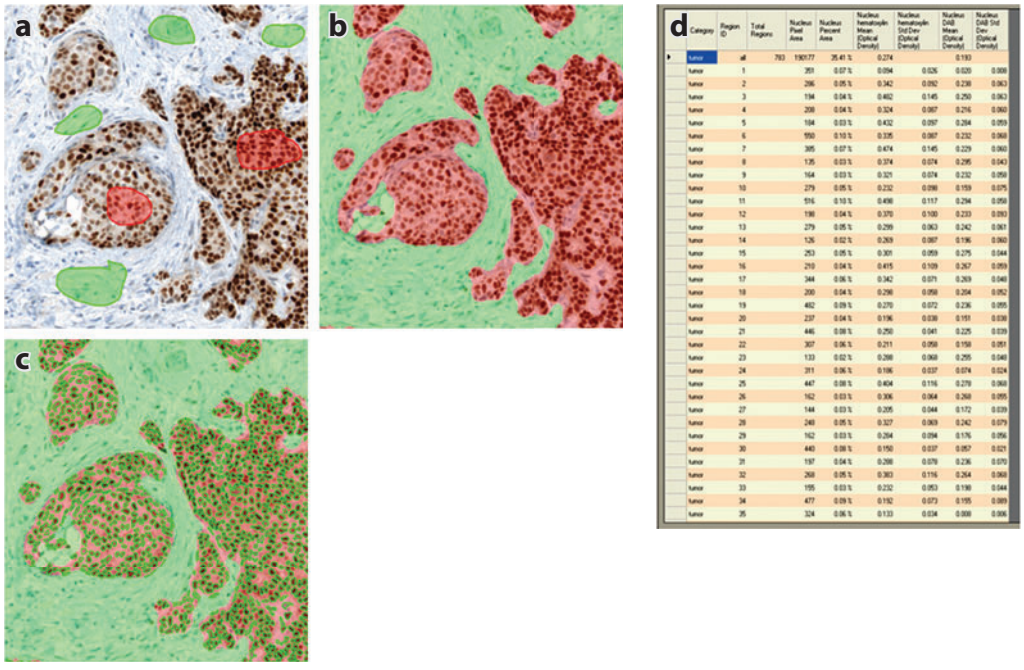

Figure 7

The inForm™ machine learning and classification system is used to score a breast carcinoma case stained for progesterone receptor. (*a*) A pathologist shows inForm a few areas of tumor (*red*) and a few areas of tumor stroma (*green*). On the basis of these training regions, (*b*) inForm learns the remaining areas with high accuracy and classifies the remainder of the image into tumor regions (*red*) and nontumor regions (*green*). (*c*) The system finds the nuclei only within the tumor region. (*d*) The system displays a list-mode data file for the optical density of diaminobenzidine for each tumor cell nucleus it finds. If more than one stain was applied to the tissue, each stain and its component intensity are associated with each cell and its subcellular localization.

In addition to open-source applications, commercial vendors are entering the quantitative analytic space. Industrial applications from Aperio, Definiens, and Perkin Elmer (formerly CRI, Inc.) have been developing cytometric image-analysis solutions. Although the software from Aperio is designed to work on whole slides, Aperio has extended its analytic platform to fluorescent images as well as spectral images that have been unmixed into spectral component planes. The software from Aperio, Definiens, and Perkin Elmer also includes region segmentation algorithms that are designed to classify regions on the basis of a user-training paradigm. During training, an experienced end user trains the segmentation algorithm by showing the software a few example regions of different disease classes (invasive tumor, in situ tumor, stroma, etc.); the algorithm then classifies the remaining image, as well as additional images that might be needed for analysis in a batch-mode study. The Aperio platform uses a genetic algorithm (Genie®) licensed from Los Alamos National Laboratory. Perkin Elmer has developed its own learn-by-example algorithm, which uses a proprietary advanced machine learning algorithm (inForm™) similar to Genie's, and Definiens has developed another proprietary train-by-example paradigm. **Figure 7** illustrates a typical training session with inForm in which a breast carcinoma was stained for ER and the tumor counterstained with hematoxylin.

IMAGE ANALYSIS

Background

WSI has led to substantial growth in the number of researchers and companies seeking

to utilize computer-based image analysis for pathology images and to develop new software tools to assist pathologists. Prior to WSI, the field of pathology image analysis was limited by pathologists' need to select fields of view upon which computer image-analysis routines could run. WSI allows the entire slide to be available for analysis; field selection can then be automated, allowing the pathologist to act as final interpreter and analyzer of the resulting data, rather than as a field selection technologist.

In general, image analysis is a multistep process that involves feature extraction, feature selection, dimensionality reduction, and classification steps. These steps are discussed in the following sections.

Feature Extraction

Research on useful features for cancer classification and diagnosis has often been inspired by grading features determined by clinicians to be particularly important for the diagnosis. The vast majority of these features are nuclear features, and many have been established as useful in the analysis of both cytopathology and histopathology imagery. Other features that assume discriminatory importance include the margin and boundary appearance of ductal, stromal, tubular, and glandular structures. Although a compilation of features for cytopathology imagery exists (95), there is relatively little such work for histopathology imagery.

Human observers' (pathologists') concept of the world is inherently object based, as opposed to the largely pixel-based representation of computer vision. As such, pathology experts describe and understand images in terms of such objects. For pathologists, diagnostic criteria are inevitably described by using cytologic terms, such as nucleus and cell, and by the relationship of larger objects to one another and to benign adjacent tissue, arrangement of glands, invasion of tissues, and desmoplastic reactions. It is therefore important to develop computer vision methods that are capable of such object-level analysis. **Figure 8** shows an automated algorithm for nuclear identification, known as nuclear segmentation in image-processing parlance.

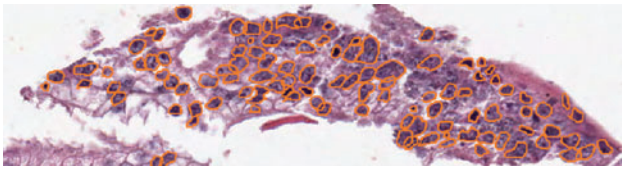

Figure 8

Automated segmentation of nuclei on a prostate histology image from a fully digitized virtual slide. In this image, the nuclei are mathematically identified by use of a combination of color, shape, and texture information. The resulting mathematical model can accurately trace out the nuclei of each cell on the basis of its digital information. Although a human can easily trace out the same nuclei, once trained a computer can perform this task in seconds; a human would take hours.

In addition to cytologic features (cells, nuclei, cell membranes), spatial relationships are used by pathologists to classify diseases. To create a set of mathematical features that relate to the spatial information that pathologists use, researchers have utilized the mathematical technique of graph theory as an effective means of representing structural and spatial information by defining a large set of topological features. Real-world graphs of various types and scales have been extensively investigated in technological, social (96), and biological (97) systems. Use of the mathematical principle of graph theory has allowed for the development of additional features from digital pathology images that can be used to model tissues and disease states. These graph-based features are quantified by definition of computable metrics. The use of graph-based spatial arrangement of histological entities (generally at low resolutions) is relatively new, especially in comparison to the wealth of research on nuclear features (at higher resolutions) that has accumulated during the same time frame. Graph-based feature extraction methods have allowed for the addition of approximately 150 new features for all graph structures (98).

Graph-based metrics can be defined and computed on a graph created by connecting the nuclei of cells to each other (i.e., a cell graph) to create a rich set of descriptive features that can be used for tissue classification. These features provide structural information about the

tissue organization, such as the distribution of local and global information around a single cell cluster or the global connectivity information of a graph. The end result of these feature extraction algorithms is a set of features that can be used for image classification. **Figure 9** illustrates graph-related features based on two computational tools: the Delaunay triangulation (**Figure 9*c***) and the minimum spanning tree (**Figure 9*d***).

Interestingly, although pathologists do not compute succinct graph-based features, pathologists often observe information about objects relative to one another, in effect using observation graph networks to understand the relationships among nuclei (for instance: Are the nuclei overlapping? Are they uniformly spaced? Are they basally oriented?) or the arrangement of glandular patterns (e.g., defining a normal lobule from an infiltrative acinar pattern). Therefore, it is unsurprising that the use of mathematically derived graph networks yields informative data about spatial information within digital pathology images.

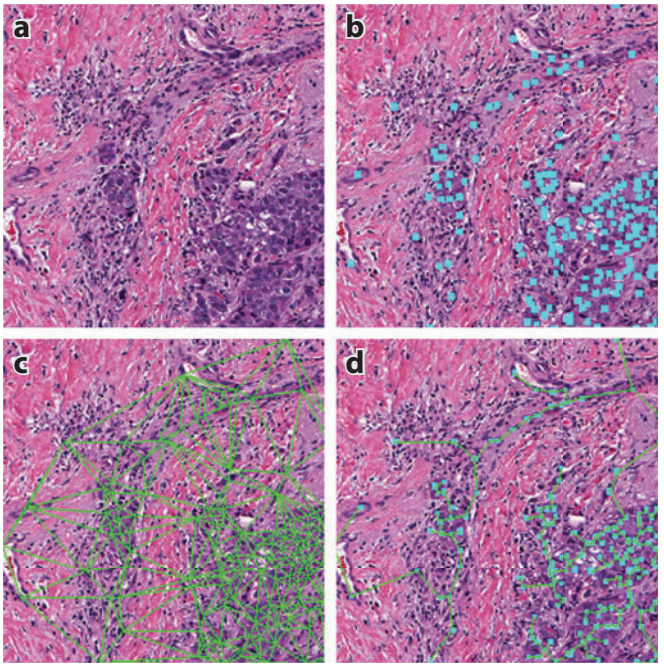

Figure 9

(*a*) Hematoxylin and eosin–stained image of breast carcinoma. (*b*) Nuclei of breast carcinoma cells identified by automated algorithm based on color deconvolution. (*c*) Delaunay triangulation calculation applied to breast cancer nuclei. (*d*) Minimum spanning tree (MST) computed on the tumor nuclei identified in panel *b*. To understand the general nature of the tissue architecture, we construct graphs such as the Delaunay triangulation and the MST. Statistics such as those related to triangle area, triangle perimeter, and MST edge length are calculated to quantify the spatial arrangement of the nuclei in the image.

Feature Selection

Although humans have innate abilities to process and understand imagery, explaining how they reach their decisions is more difficult; pathologists often rely on a small set of features that occur at a high frequency within an image scene to classify disease states and patterns. As such, image-analysis applications often begin with large feature sets that are generated in the hopes that some subset of features incorporates the information used by the human expert for analysis. Therefore, many of the generated features could be redundant or irrelevant. Feature selection is a way to extract the relevant and important features from a large set of features.

Feature selection in histopathological image analysis provides several benefits in addition to improving accuracy. Because images tend to be relatively large, one should calculate a small subset of features, which reduces the computational complexity of classification algorithms. A smaller number of features also makes it easier to explain the underlying model and to improve the chances of generalization of the developed system. Additionally, in a multiresolution framework (e.g., a whole digital slide with varying levels of magnification), a set of features that are useful at a given resolution may not be relevant at another resolution, even within the same image. A feature selection algorithm helps determine which features should be used at a given resolution. This fact is intuitively obvious to the practicing pathologist, who uses scale-based information naturally and seamlessly. Who hasn't opened a slide tray and moved immediately to the diagnostic slide by looking for the bluest slide? At very low magnification (scale), color information is very informative, whereas at higher

magnification, other features of architecture and nuclear morphology become important.

An optimal feature selection method requires an exhaustive search, which is not practical for a large set of features generated from a large data set. Therefore, several heuristic algorithms that use classification accuracy as the optimality criterion have been developed and used in various image-processing strategies. (14–16, 99–101).

Dimensionality Reduction

Whereas feature selection aims to choose those features (and reduce the feature dimensionality) that best optimize some criterion related to the class labels (i.e., that separate different classes or states of disease) of the data (e.g., classification performance), dimensionality reduction techniques aim to reduce the overall dimensionality of the data set from thousands of features to a smaller set of features on the basis of some other criterion. A well-known and commonly used method of linear dimensionality reduction is principal components analysis (PCA).

PCA (103) attempts to find a new coordinate system that shows the first three axes that define the maximum variance of the data. The first feature (eigenvector) is incorporated in the first dimension, the next-largest eigenvector holds the next-largest amount of variance in the starting high-dimensional data set, and so forth. By reducing the dimensionality of the starting problems from a feature space with thousands of features to three features, one can more easily visualize the data and their relationship to the outcome variable. In this lower-dimensional feature space, classification algorithms can be run to separate clinical or biological conditions. Thus, when only the first few dimensions of the PCA transform are retained, the sources of the largest amount of variation in the data are maintained.

Recently, nonlinear dimensionality reduction (NLDR) methods have become popular in learning applications. These methods overcome a major limitation of linear dimensionality reduction methods, such as PCA, which assume that the geometrical structure of the high-dimensional feature space is a linear or straight-line relationship. PCA is a linear transformation that transforms the data to a new coordinate system such that the direction with the greatest variance lies on the first coordinate (termed the first principal component), the second greatest variance on the second coordinate, and so on. In reality, high-dimensional feature spaces are often composed of highly nonlinear structures, and locality-preserving dimensionality reduction methods are highly sought. Several manifold learning algorithms have been constructed to deal with different types of data (104–108). Graph embedding is one such algorithm that aims to nonlinearly project high-dimensional data into a reduced dimensional space while preserving object adjacencies (109–111). The high-dimensional feature space is significantly reduced, in terms of the number of dimensions, to a lower-dimensional feature vector space. A key value of NLDR methods is that they preserve object adjacencies. Thus, if two objects (e.g., pathology images) are close to one another in the original high-dimensional feature space, they will likewise be embedded close to one another in the lower-dimensional subspace. This preservation of feature adjacency suggests that the two objects are similar to one another, perhaps in terms of biological or clinical potential (survival, nuclear grade, etc.).

Classification

Following feature selection extraction, feature selection, and dimensionality reduction, classification schema can be run on image data to classify the features into clinical classes (such as different histologic states, different tumor grades, and different outcome measures). Alternately, classification can be performed on the large feature space, but doing so makes for a computationally challenging problem. Unlike some other applications of image analysis, in histopathology imagery a primary consideration in the choice of a classifier is its ability to deal with large, highly dense data sets. Also, due to multiple image scales at which relevant

information may be extracted from histological imagery, use of an ensemble (combination) of classifiers as opposed to a single classifier has been proposed. Following feature extraction, selection, and dimensionality reduction, different schemas for classification may be applied to the histopathologic images.

Multiclassifier Ensemble Schemes

Both theoretical and empirical results have established that, in terms of accuracy, ensembles of classifiers generally outperform monolithic solutions. Learning ensembles or multiple classifier systems are methods for improving classification accuracy through aggregation of several similar classifiers' predictions and, thereby, reducing either the bias or the variance of the individual classifiers (112–114).

Support vector machines Support vector machines (SVMs) project a set of training data, *E*, that represents two different classes into a high-dimensional space by means of a kernel function, *K*. In this transformed data space, nonlinear data are transformed so that a flat line can be generated (a discriminating hyperplane) to separate the classes so as to maximize the class separation. Testing data are then projected into the high-dimensional space via *K*, and the test data are classified on the basis of where they fall with respect to the hyperplane. The kernel function *K* defines the method in which data are projected into the high-dimensional space. A commonly used kernel known as the radial basis function has been employed to distinguish among three different classes of prostate tissue (115), as well as to differentiate colon adenocarcinoma histopathology images from benign histopathology images (116) and to classify four different subtypes of meningiomas from their histopathology images (117).

AdaBoost The AdaBoost algorithm for classification is used to combine a number of weak classifiers (image features that do not individually sort images into different object classes) to generate a strong classifier (a combined classifier made by linearly combining and weighting weak classifiers). A study by Doyle et al. (118) presented a hierarchical boosted cascade scheme (a linear combination of features created through the selected weighting of individual features to best classify an image) for detecting suspicious areas on digitized prostate histopathology. Efficient and accurate analysis is performed by first detecting those areas found to be suspicious only at lower scales (low magnification). Analysis at subsequent, higher magnifications is limited to those regions deemed to be suspicious at lower scales. Pixels classified as nontumor at a lower magnification (scale) are discarded at the subsequent higher scale, which reduces the number of pixels needed for analysis at higher scales. The process is repeated using an increasingly large number of image features and an increasing classification threshold at each iteration.

Case Study: Prostate Carcinoma Grading

Classification of histopathology images is often the ultimate goal in image analysis, particularly in cancer applications. Features derived from segmented nuclei and glands from histopathology are usually a prerequisite to extraction of higher-level information regarding the state of the disease. For instance, the grading of prostate cancer by Jafari-Khouzani & Soltanian-Zadeh (119) yielded 97% accuracy for H&E-stained imagery on the basis of features derived from nuclear structures in histopathology. Tabesh et al. (120) found 96.7% accuracy in discriminating between prostate tissue slides with cancer and without cancer, and they found 81% accuracy in the discrimination between low and high Gleason grades. Using nonlinear dimensionality and SVMs, Madabhushi et al. (121) demonstrated 95.8% classification accuracy between Gleason grade 3 and grade 4, 96% accuracy between Gleason grade 3 and benign, and 100% accuracy between Gleason grade 4 and benign.

Case Study: Predicting Breast Carcinoma Outcomes

In contrast to prostate cancer grading, in which a computer is trained to mimic the effort of a pathologist (i.e., to identify and grade cancer by using predefined human categories), Beck et al. (122) used an unbiased image-analysis solution that they developed, named C-Path, to identify a feature set that predicted 5-year survival of breast carcinoma patients. Tissue microarrays (TMAs) of breast carcinoma were analyzed in the Definiens Developer XD™ software platform, and a feature set of some 6,000 image features were defined and measured. This feature set included both epithelial and stromal features. Using a machine learning algorithm (L1 logistic regression), Beck et al. developed a prediction model that accurately predicts good- versus poor-prognosis patients and is independent of molecular subtype, stage, ER status, and pathology grade. This model was trained on a TMA from the Netherlands Cancer Institute and was validated on a separate breast cancer TMA from Vancouver General Hospital. One of the unique features of this study was the identification of stromal features that are strongly prognostic, are not routinely examined, and are scored in routine pathologic analysis of breast cancer samples. This study underscores the power of automated and unbiased image-analysis and machine learning systems and points toward an exciting opportunity for future studies in this area.

CONCLUSIONS

From its genesis as an interesting idea in the late 1990s, digital pathology has become a useful and valuable tool in clinical and research pathology. This transition was initially fueled by the development of digital slide scanners, fluorescent slide scanners, multispectral imaging hardware, and computational horsepower. Today, integrated systems with increasingly complex and functional software tools are being developed and will become part of our diagnostic toolbox as we move into personalized medicine. Of the various barriers to widespread adoption that were described above, comprehensive validation of this technology for diagnostic purposes across the complete spectrum of surgical pathology represents the most important. As this process unfolds, digital pathology will undoubtedly open up new avenues for computational exploration of individual disease tissues and will transform the practice of pathology.

DISCLOSURE STATEMENT

A.E. is a nonpaid member of the Omnyx Collaborators' Program. The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

LITERATURE CITED

1. Ho J, Parwani AV, Jukic DM, Yagi Y, Anthony L, Gilbertson JR. 2006. Use of whole slide imaging in surgical pathology quality assurance: design and pilot validation studies. *Hum. Pathol.* 37:322–31
2. Pantanowitz L. 2010. Digital images and the future of digital pathology. *J. Pathol. Inform.* 1:15
3. Weinstein RS, Graham AR, Richter LC, Barker GP, Krupinski EA, et al. 2009. Overview of telepathology, virtual microscopy, and whole slide imaging: prospects for the future. *Hum. Pathol.* 40:1057–69
4. Gilbertson JR, Ho J, Anthony L, Jukic DM, Yagi Y, Parwani AV. 2006. Primary histologic diagnosis using automated whole slide imaging: a validation study. *BMC Clin. Pathol.* 6:4
5. Williams S, Henricks WH, Becich MJ, Toscano M, Carter AB. 2010. Telepathology for patient care: What am I getting myself into? *Adv. Anat. Pathol.* 17:130–49

6. Della Mea V, Bortolotti N, Beltrami CA. 2009. eSlide suite: an open source software system for whole slide imaging. *J. Clin. Pathol.* 62:749–51
7. Evans AJ, Chetty R, Clarke BA, Croul S, Ghazarian DM, et al. 2009. Primary frozen section diagnosis by robotic microscopy and virtual slide telepathology: the University Health Network experience. *Hum. Pathol.* 40:1070–81
8. Massone C, Brunasso AM, Campbell TM, Soyer HP. 2008. State of the art of teledermatopathology. *Am. J. Dermatopathol.* 30:446–50
9. Difranco MD, O'Hurley G, Kay EW, Watson RW, Cunningham P. 2011. Ensemble based system for whole-slide prostate cancer probability mapping using color texture features. *Comput. Med. Imaging Graph.* 35:629–45
10. Gabril MY, Yousef GM. 2010. Informatics for practicing anatomical pathologists: marking a new era in pathology practice. *Mod. Pathol.* 23:349–58
11. Patterson ES, Rayo M, Gill C, Gurcan MN. 2011. Barriers and facilitators to adoption of soft copy interpretation from the user perspective: lessons learned from filmless radiology for slideless pathology. *J. Pathol. Inform.* 2:9
12. Huisman A, Looijen A, van den Brink SM, van Diest PJ. 2010. Creation of a fully digital pathology slide archive by high-volume tissue slide scanning. *Hum. Pathol.* 41:751–57
13. Siegel EL, Kolonder RM. 1999. *Filmless Radiology*. New York: Springer
14. Dee FR. 2009. Virtual microscopy in pathology education. *Hum. Pathol.* 40:1112–21
15. Hedvat CV. 2010. Digital microscopy: past, present, and future. *Arch. Pathol. Lab. Med.* 134:1666–70
16. Thorstenson S. 2009. *From the conventional microscope to the digital slide scanner in routine diagnostic histopathology*. Presented at Pathol. Vis. Conf., San Diego
17. Costello SS, Johnston DJ, Dervan PA, O'Shea DG. 2003. Development and evaluation of the virtual pathology slide: a new tool in telepathology. *J. Med. Internet Res.* 5:e11
18. Krupinski EA, Tillack AA, Richter L, Henderson JT, Bhattacharyya AK, et al. 2006. Eye-movement study and human performance using telepathology virtual slides: implications for medical education and differences with experience. *Hum. Pathol.* 37:1543–56
19. Stratman C. 2009. *Digital pathology in the clinical histology lab: a time and motion study.* Presented at Pathol. Vis. Conf., San Diego
20. Velez N, Jukic D, Ho J. 2008. Evaluation of two whole-slide imaging applications in dermatopathology. *Hum. Pathol.* 39:1341–49
21. Bautista PA, Yagi Y. 2010. Improving the visualization and detection of tissue folds in whole slide images through color enhancement. *J. Pathol. Inform.* 1:25
22. Evered A, Dudding N. 2010. Accuracy and perceptions of virtual microscopy compared with glass slide microscopy in cervical cytology. *Cytopathology* 22:82–87
23. Kalinski T, Zwonitzer R, Sel S, Evert M, Guenther T, et al. 2008. Virtual 3D microscopy using multiplane whole slide images in diagnostic pathology. *Am. J. Clin. Pathol.* 130:259–64
24. Thrall M, Pantanowitz L, Khalbuss W. 2011. Telecytology: clinical applications, current challenges, and future benefits. *J. Pathol. Inform.* 2:51
25. Schrader T, Niepage S, Leuthold T, Saeger K, Schluns K, et al. 2006. The diagnostic path, a useful visualisation tool in virtual microscopy. *Diagn. Pathol.* 1:40
26. DICOM Stand. Comm. Work. Group 26 Pathol. 2008. *Specimen Module and Revised Pathology SOP Classes*, suppl. 122. Rosslyn, VA: DICOM. **ftp://medical.nema.org/medical/dicom/final/sup122_ft2.pdf**
27. DICOM Stand. Comm. Work. Group 26 Pathol. *Whole Slide Microscopic Image IOD and SOP Classes*, suppl. 145. Rosslyn, VA: DICOM. **ftp://medical.nema.org/medical/dicom/supps/sup145_ft.pdf**.
28. Daniel C, Rojo MG, Klossa J, Della Mea V, Booker D, et al. 2011. Standardizing the use of whole slide images in digital pathology. *Comput. Med. Imaging Graph.* 35:496–505
29. Krupinski EA. 2009. Virtual slide telepathology workstation of the future: lessons learned from teleradiology. *Hum. Pathol.* 40:1100–11
30. McClintock DS, Lee RE, Gilbertson JR. 2012. Using computerized workflow simulations to assess the feasibility of whole slide imaging full adoption in a high-volume histology laboratory. *Anal. Cell. Pathol.* 35:57–64

31. Isaacs M, Lennerz JK, Yates S, Clermont W, Rossi J, Pfeifer JD. 2011. Implementation of whole slide imaging in surgical pathology: a value added approach. *J. Pathol. Inform.* 2:39
32. Evans AJ. 2011. Re: Barriers and facilitators to adoption of soft copy interpretation from the user perspective: lessons learned from filmless radiology for slideless pathology. *J. Pathol. Inform.* 2:8
33. Evans AJ, Sinard JH, Fatheree LA, Henricks WH, Carter AB, et al. 2011. Validating whole slide imaging for diagnostic purposes in pathology: recommendations of the College of American Pathologists (CAP) Pathology and Laboratory Quality Center. *Anal. Cell. Pathol.* 34:169–203
34. Jukic DM, Drogowski LM, Martina J, Parwani AV. 2011. Clinical examination and validation of primary diagnosis in anatomic pathology using whole slide digital images. *Arch. Pathol. Lab. Med.* 135:372–78
35. Mooney E, Hood AF, Lampros J, Kempf W, Jemec GB. 2011. Comparative diagnostic accuracy in virtual dermatopathology. *Skin Res. Technol.* 17:251–55
36. Chargari C, Comperat E, Magne N, Vedrine L, Houlgatte A, et al. 2011. Prostate needle biopsy examination by means of virtual microscopy. *Pathol. Res. Pract.* 207:366–69
37. Fine JL, Grzybicki DM, Silowash R, Ho J, Gilbertson JR, et al. 2008. Evaluation of whole slide image immunohistochemistry interpretation in challenging prostate needle biopsies. *Hum. Pathol.* 39:564–72
38. Tsuchihashi Y, Takamatsu T, Hashimoto Y, Takashima T, Nakano K, Fujita S. 2008. Use of virtual slide system for quick frozen intra-operative telepathology diagnosis in Kyoto, Japan. *Diagn. Pathol.* 3(Suppl. 1):6
39. Fallon MA, Wilbur DC, Prasad M. 2010. Ovarian frozen section diagnosis: Use of whole-slide imaging shows excellent correlation between virtual slide and original interpretations in a large series of cases. *Arch. Pathol. Lab. Med.* 134:1020–23
40. Nielsen PS, Lindebjerg J, Rasmussen J, Starklint H, Waldstrom M, Nielsen B. 2010. Virtual microscopy: an evaluation of its validity and diagnostic performance in routine histologic diagnosis of skin tumors. *Hum. Pathol.* 41:1770–76
41. Ramey JP, Fung K, Hassell LA. 2011. Validation of pathologist use of whole slide images for remote frozen section evaluation. *Mod. Pathol.* 24:436 (Abstr.)
42. Ramey JP, Fung K, Hassell LA. 2011. Use of mobile high resolution device for remote frozen section evaluation of whole slide images. *Mod. Pathol.* 24:341 (Abstr.)
43. Reyes C, Ikpatt F, Nadiji M, Cote RJ. 2011. Is virtual microscopy ready for the prime time? A comparison with conventional microscopy. *Mod. Pathol.* 24:454 (Abstr.)
44. Ho J. 2009. *Validating digital slides for clinical use: When is image quality good enough?* Presented at Pathol. Vis. Conf., San Diego
45. Sawai T, Uzuki M, Kamataki A, Tofukuji I. 2010. The state of telepathology in Japan. *J. Pathol. Inform.* 1:13
46. Rodriguez-Urrego PA, Cronin AM, Al-Ahmadie HA, Gopalan A, Tickoo SK, et al. 2011. Interobserver and intraobserver reproducibility in digital and routine microscopic assessment of prostate needle biopsies. *Hum. Pathol.* 42:68–74
47. Wilbur DC, Madi K, Colvin RB, Duncan LM, Faquin WC, et al. 2009. Whole-slide imaging digital pathology as a platform for teleconsultation: a pilot study using paired subspecialist correlations. *Arch. Pathol. Lab. Med.* 133:1949–53
48. Bloom K. 2009. *The virtual consultative network*. Presented at Pathol. Vis. Conf., San Diego
49. Spinosa JC. 2008. Minute by minute, digital a boon to tumor board. *Coll. Am. Pathol. Today* 2008 Arch.:Oct.
50. Hede K. 2008. Breast cancer testing scandal shines spotlight on black box of clinical laboratory testing. *J. Natl. Cancer Inst.* 100:836–44
51. Lopez AM, Graham AR, Barker GP, Richter LC, Krupinski EA, et al. 2009. Virtual slide telepathology enables an innovative telehealth rapid breast care clinic. *Hum. Pathol.* 40:1082–91
52. Raab SS, Nakhleh RE, Ruby SG. 2005. Patient safety in anatomic pathology: measuring discrepancy frequencies and causes. *Arch. Pathol. Lab. Med.* 129:459–66
53. Manion E, Cohen MB, Weydert J. 2008. Mandatory second opinion in surgical pathology referral material: clinical consequences of major disagreements. *Am. J. Surg. Pathol.* 32:732–37
54. Raab SS, Grzybicki DM, Mahood LK, Parwani AV, Kuan SF, Rao UN. 2008. Effectiveness of random and focused review in detecting surgical pathology error. *Am. J. Clin. Pathol.* 130:905–12

55. Woolgar JA, Ferlito A, Devaney KO, Rinaldo A, Barnes L. 2011. How trustworthy is a diagnosis in head and neck surgical pathology? A consideration of diagnostic discrepancies (errors). *Eur. Arch. Otorhinolaryngol.* 268:643–51
56. Graham AR, Bhattacharyya AK, Scott KM, Lian F, Grasso LL, et al. 2009. Virtual slide telepathology for an academic teaching hospital surgical pathology quality assurance program. *Hum. Pathol.* 40:1129–36
57. Hassell LA, Fung KM, Chaser B. 2011. Digital slides and ACGME resident competencies in anatomic pathology: an altered paradigm for acquisition and assessment. *J. Pathol. Inform.* 2:27
58. Bruch LA, De Young BR, Kreiter CD, Haugen TH, Leaven TC, Dee FR. 2009. Competency assessment of residents in surgical pathology using virtual microscopy. *Hum. Pathol.* 40:1122–28
59. Fonyad L, Gerely L, Cserneky M, Molnar B, Matolcsy A. 2010. Shifting gears higher—digital slides in graduate education—4 years experience at Semmelweis University. *Diagn. Pathol.* 5:73
60. Li L, Dangott BJ, Parwani AV. 2010. Development and use of a genitourinary pathology digital teaching set for trainee education. *J. Pathol. Inform.* 1:2
61. Marchevsky AM, Khurana R, Thomas P, Scharre K, Farias P, Bose S. 2006. The use of virtual microscopy for proficiency testing in gynecologic cytopathology: a feasibility study using ScanScope. *Arch. Pathol. Lab. Med.* 130:349–55
62. Stewart J 3rd, Miyazaki K, Bevans-Wilkins K, Ye C, Kurtycz DF, Selvaggi SM. 2007. Virtual microscopy for cytology proficiency testing: Are we there yet? *Cancer* 111:203–9
63. Fung KM, Hassell LA, Talbert ML, Wiechmann AF, Chaser BE, Ramey J. 2012. Whole slide images and digital media in pathology education, testing, and practice: the Oklahoma experience. *Anal. Cell. Pathol.* 35:37–40
64. van den Tweel JG, Bosman FT. 2011. The use of virtual slides in the EUROPALS examination. *Diagn. Pathol.* 6(Suppl. 1):23
65. Merk M, Knuechel R, Perez-Bouza A. 2010. Web-based virtual microscopy at the RWTH Aachen University: didactic concept, methods and analysis of acceptance by the students. *Ann. Anat.* 192:383–87
66. Paulsen FP, Eichhorn M, Brauer L. 2010. Virtual microscopy: the future of teaching histology in the medical curriculum? *Ann. Anat.* 192:378–82
67. Collier L, Dunham S, Braun MW, O'Loughlin VD. 2012. Optical versus virtual: teaching assistant perceptions of the use of virtual microscopy in an undergraduate human anatomy course. *Anat. Sci. Educ.* 5:10–19
68. Farah CS, Maybury TS. 2009. The e-evolution of microscopy in dental education. *J. Dent. Educ.* 73:942–49
69. Helin H, Lundin M, Lundin J, Martikainen P, Tammela T, et al. 2005. Web-based virtual microscopy in teaching and standardizing Gleason grading. *Hum. Pathol.* 36:381–86
70. Lundin M, Lundin J, Helin H, Isola J. 2004. A digital atlas of breast histopathology: an application of Web based virtual microscopy. *J. Clin. Pathol.* 57:1288–91
71. Angeletti C, Harvey NR, Khomitch V, Fischer AH, Levenson RM, Rimm DL. 2005. Detection of malignancy in cytology specimens using spectral-spatial analysis. *Lab. Investig.* 85:1555–64
72. Jaganath R, Angeletti C, Levenson RM, Rimm DL. 2004. Diagnostic classification of urothelial cells in urine cytology specimens using exclusively spectral information. *Cancer* 102:186–91
73. Mansoor I, Zalles C, Zahid F, Gossage K, Levenson RM, Rimm DL. 2008. Fine-needle aspiration of follicular adenoma versus parathyroid adenoma: the utility of multispectral imaging in differentiating lesions with subtle cytomorphologic differences. *Cancer* 114:22–26
74. Levenson RM. 2006. Spectral imaging perspective on cytomics. *Cytometry A* 69:592–600
75. Levenson RM, Lynch DT, Kobayashi H, Backer JM, Backer MV. 2008. Multiplexing with multispectral imaging: from mice to microscopy. *Inst. Lab. Anim. Res. J.* 49:78–88
76. Levenson RM, Mansfield JR. 2006. Multispectral imaging in biology and medicine: slices of life. *Cytometry A* 69:748–58
77. Mansfield JR, Gossage KW, Hoyt CC, Levenson RM. 2005. Autofluorescence removal, multiplexing, and automated analysis methods for in-vivo fluorescence imaging. *J. Biomed. Opt.* 10:41207
78. Al-Kofahi Y, Lassoud W, Grama K, Nath S, Zhu J, et al. 2011. Cell-based quantification of molecular biomarkers in histopathology specimens. *Histopathology* 59:40–54

79. Al-Kofahi Y, Lassoued W, Lee W, Roysam B. 2010. Improved automatic detection and segmentation of cell nuclei in histopathology images. *IEEE Trans. Biomed. Eng.* 57:841–52
80. Levenson RM. 2004. Spectral imaging and pathology: seeing more. *Lab. Med.* 4:244–51
81. Dicker DT, Lerner J, Van Belle P, Barth SF, Guerry D, et al. 2006. Differentiation of normal skin and melanoma using high resolution hyperspectral imaging. *Cancer Biol. Ther.* 5:1033–38
82. Keshava N. 2003. A survey of spectral unmixing algorithms. *Lincoln Lab. J.* 14:55–78
83. Woolfe F, Gerdes M, Bello M, Tao X, Can A. 2010. Autofluorescence removal by non-negative matrix factorization. *IEEE Trans. Image Process* 20:1085–93
84. Moeder CB, Giltnane JM, Moulis SP, Rimm DL. 2009. Quantitative, fluorescence-based in situ assessment of protein expression. *Methods Mol. Biol.* 520:163–75
85. Dolled-Filhart M, Gustavson M, Camp RL, Rimm DL, Tonkinson JL, Christiansen J. 2010. Automated analysis of tissue microarrays. *Methods Mol. Biol.* 664:151–62
86. Menard S, Balsari A, Tagliabue E, Camerini T, Casalini P, et al. 2008. Biology, prognosis and response to therapy of breast carcinomas according to HER2 score. *Ann. Oncol.* 19:1706–12
87. Chivukula M, Bhargava R, Brufsky A, Surti U, Dabbs DJ. 2008. Clinical importance of HER2 immunohistologic heterogeneous expression in core-needle biopsies versus resection specimens for equivocal (immunohistochemical score 2+) cases. *Mod. Pathol.* 21:363–68
88. Kurosumi M. 2007. Immunohistochemical assessment of hormone receptor status using a new scoring system (J-score) in breast cancer. *Breast Cancer* 14:189–93
89. Camp RL, Chung GG, Rimm DL. 2002. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat. Med.* 8:1323–27
90. Ryan D, Mulrane L, Rexhepaj E, Gallagher WM. 2011. Tissue microarrays and digital image analysis. *Methods Mol. Biol.* 691:97–112
91. Mulrane L, Rexhepaj E, Penney S, Callanan JJ, Gallagher WM. 2008. Automated image analysis in histopathology: a valuable tool in medical diagnostics. *Expert Rev. Mol. Diagn.* 8:707–25
92. Taylor CR, Levenson RM. 2006. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment. II. *Histopathology* 49:411–24
93. Anagnostou VK, Welsh AW, Giltnane JM, Siddiqui S, Liceaga C, et al. 2010. Analytic variability in immunohistochemistry biomarker studies. *Cancer Epidemiol. Biomark. Prev.* 19:982–91
94. Emerson JW, Dolled-Filhart M, Harris L, Rimm DL, Tuck DP. 2009. Quantitative assessment of tissue biomarkers and construction of a model to predict outcome in breast cancer using multiple imputation. *Cancer Inform.* 7:29–40
95. Rodenacker K, Bengtsson E. 2003. A feature set for cytometry on digitized microscopic images. *Anal. Cell Pathol.* 25:1–36
96. Watts DJ, Strogatz SH. 1998. Collective dynamics of "small-world" networks. *Nature* 393:440–42
97. Wuchty S, Stadler PF. 2003. Centers of complex networks. *J. Theor. Biol.* 223:45–53
98. Boucheron LE, Harvey NR, Manjunath BS. 2008. *On using cytoplasm and stroma features in automated cancer diagnosis*. Presented at Workshop Bio-Image Inform. Biol. Imaging, Comput. Vis. Data Min., Santa Barbara
99. Peng Y, Wu Z, Jiang J. 2009. A novel feature selection approach for biomedical data classification. *J. Biomed. Inform.* 43:15–23
100. Sima C, Dougherty ER. 2006. What should be expected from feature selection in small-sample settings. *Bioinformatics* 22:2430–36
101. Xiong M, Fang X, Zhao J. 2001. Biomarker identification by feature wrappers. *Genome Res.* 11:1878–87
102. Jain A, Zongker D. 1997. Feature selection: evaluation, application, and small sample performance. *IEEE Trans. Pattern Anal. Mach. Intell.* 19:153–58
103. Jolliffe IT. 2002. *Principal Component Analysis*. New York: Springer
104. Qiao H, Zhang P, Zhang B, Zheng S. 2009. Learning an intrinsic-variable preserving manifold for dynamic visual tracking. *IEEE Trans. Syst. Man Cybern. B* 40:868–80
105. Takahashi S, Fujishiro I, Okada M. 2009. Applying manifold learning to plotting approximate contour trees. *IEEE Trans. Vis. Comput. Graph.* 15:1185–92
106. Hou Y, Zhang P, Xu X, Zhang X, Li W. 2009. Nonlinear dimensionality reduction by locally linear inlaying. *IEEE Trans. Neural Netw.* 20:300–15

107. Li X, Lin S, Yan S, Xu D. 2008. Discriminant locally linear embedding with high-order tensor data. *IEEE Trans. Syst. Man Cybern. B* 38:342–52
108. Law MH, Jain AK. 2006. Incremental nonlinear dimensionality reduction by manifold learning. *IEEE Trans. Pattern Anal. Mach. Intell.* 28:377–91
109. Madabhushi A, Yang P, Rosen M, Weinstein S. 2006. Distinguishing lesions from posterior acoustic shadowing in breast ultrasound via non-linear dimensionality reduction. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 1:3070–73
110. Yan S, Xu D, Zhang B, Zhang HJ, Yang Q, Lin S. 2007. Graph embedding and extensions: a general framework for dimensionality reduction. *IEEE Trans. Pattern Anal. Mach. Intell.* 29:40–51
111. Madabhushi A, Shi J, Rosen M, Tomaszeweski JE, Feldman MD. 2005. Graph embedding to improve supervised classification and novel class detection: application to prostate cancer. *Med. Image Comput. Comput. Assist. Interv.* 8:729–37
112. Kuncheva LI. 1993. An aggregation of pro and con evidence for medical decision support systems. *Comput. Biol. Med.* 23:417–24
113. Kuncheva LI. 1991. Evaluation of computerized medical diagnostic decisions via fuzzy sets. *Int. J. Biomed. Comput.* 28:91–100
114. Kuncheva LI. 1990. Fuzzy multi-level classifier for medical applications. *Comput. Biol. Med.* 20:421–31
115. Doyle S, Hwang M, Shah K, Madabhushi A, Tomaszeweski J, Feldman M. 2007. Automated grading of prostate cancer using architectural and textural image features. *Int. Symp. Biomed. Imaging*, pp. 1284–87
116. Rajpoot K, Rajpoot N. 2004. SVM optimization for hyperspectral colon tissue cell classification. *Med. Image Comput. Comput. Assist. Interv.* 2:829–37
117. Qureshi HNR, Wilson R, Nattkemper T, Hans V. 2007. *Comparative analysis of discriminant wavelet packet features and raw image features for classification of meningioma subtypes*. Presented at Med. Image Underst. Anal., Aberystwyth, Wales
118. Doyle S, Madabhushi A, Feldman M, Tomaszeweski J. 2006. A boosting cascade for automated detection of prostate cancer from digitized histology. *Med. Image Comput. Comput. Assist. Interv.* 9:504–11
119. Jafari-Khouzani K, Soltanian-Zadeh H. 2003. Multiwavelet grading of pathological images of prostate. *IEEE Trans. Biomed. Eng.* 50:697–704
120. Tabesh A, Teverovskiy M, Pang HY, Kumar VP, Verbel D, et al. 2007. Multifeature prostate cancer diagnosis and Gleason grading of histological images. *IEEE Trans. Med. Imaging* 26:1366–78
121. Madabhushi A, Doyle S, Lee G, Basavanhally A, Monaco J, et al. 2010. Integrated diagnostics: a conceptual framework with examples. *Clin. Chem. Lab. Med.* 48:989–98
122. Beck AH, Sangoi AR, Leung S, Marinelli RJ, Nielsen TO, et al. 2011. Systematic analysis of breast cancer morphology uncovers stromal features associated with survival. *Sci. Transl. Med.* 3:108ra13

Annual Review of Pathology: Mechanisms of Disease

Volume 8, 2013

Contents

Indexes

Errata

An online log of corrections to *Annual Review of Pathology: Mechanisms of Disease* articles may be found at http://pathol.annualreviews.org