Validation of novel optical imaging technologies: the pathologists' view

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Abstract. Noninvasive optical imaging technology has the potential to improve the accuracy of disease detection and predict treatment response. Pathology provides the critical link between the biological basis of an image or spectral signature and clinical outcomes obtained through optical imaging. The validation of optical images and spectra requires both morphologic diagnosis from histopathology and parametric analysis of tissue features above and beyond the declared pathologic "diagnosis." Enhancement of optical imaging modalities with exogenously applied biomarkers also requires validation of the biological basis for molecular contrast. For an optical diagnostic or prognostic technology to be useful, it must be clinically important, independently informative, and of demonstrated beneficial value to patient care. Its usage must be standardized with regard to methods, interpretation, reproducibility, and reporting, in which the pathologist plays a key role. By providing insight into disease pathobiology, interpretive or quantitative analysis of tissue material, and expertise in molecular diagnosis, the pathologist should be an integral part of any team that is validating novel optical imaging modalities. This review will consider (1) the selection of validation biomarkers; (2) standardization in tissue processing, diagnosis, reporting, and quantitative analysis; (3) the role of the pathologist in study design; and (4) reference standards, controls, and interobserver variability. [DOI: 10.1117/1.2795569]

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1 Introduction

The promise of novel biomedical imaging methods, such as optical imaging, is not only to increase the frequency and accuracy with which disease can be detected but also to improve the prediction and monitoring of disease progression or regression during treatment. A critical component of research seeking to develop new imaging concepts is validation of the image signal itself. This requires establishing the biological basis for image contrast and depends critically on achievable signal-to-background ratios. Over and above correlation of optical images with tissue morphology, there must also be validation of parametric variables obtained through optical methodologies, and validation of exogenously applied biomarkers with regards to their tissue specificity and molecular targets. In each of these instances, pathology provides the validation.

Optical imaging research groups need to develop associations between image data and standardized biophysical markers (histopathologic, morphometric, proteomic, genomic) to identify the pathologic measures that represent the most promising and appropriate surrogates for new image indices. Use of parametric indices derived from pathologic analysis of tissue specimens will ensure a standardized approach for image interpretation and spectral analysis. Such standardization will be a critical foundation for the future multicenter imaging

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Table 1 Utilization of pathology "standards" by NTROI principals.

Requirement	Application
Diagnosis	By biopsy, following *in vivo* optical imaging
	By biopsy, following *ex vivo* optical imaging
	Of excised tissue, correlated with previous *in vivo* optical imaging
	Of excised tissue, correlated with *ex vivo* optical imaging
Morphology	Tumor histology and immunohistochemistry
	Tissue parametric properties (see Table 2)
Spectroscopy	Of tissue *in situ*, followed by pathology diagnosis
	Of tissue sections, with direct morphologic correlation
	Of intact excised tissue, followed by oriented morphologic analysis

trials that are essential for adoption of novel imaging techniques into medical practice, because tissue analysis will be performed by a broader set of collaborating pathologists.

The Network for Translational Research in Optical Imaging (NTROI), funded by the National Institutes of Health (NIH) [through the National Cancer Institute (NCI)], is a national initiative composed of networks of researchers seeking to translate new optical imaging technologies in useful modalities for different organ sites (breast, esophagus, colon). NTROI includes the Pathology Working Group (the authors of this paper), which is composed of named collaborating pathologists with input from the National Institutes of Standards and Technology (NIST). The mission of the Pathology Working Group is to develop the pathology standards necessary for support of the optical imaging research being performed within NTROI. The current NTROI utilization of pathology is given in Table 1. The following review highlights the approach used by the Pathology Working Group to validate optical imaging standards for NTROI.

2 Current Approaches to Selecting Validating Biomarkers

One of the key applications of optical imaging is to identify the salient properties of human tissue that mark the presence of disease (usually neoplasia) *and* exhibit optical contrast. In the simplest sense, these tissue parameters constitute "biomarkers" of disease. The selection of potential biomarkers that signify tissue disease should be driven by the molecular basis of emerging technologies.[1] For example, near-infrared (NIR) spectroscopy quantifies hemoglobin and deoxyhemoglobin concentrations and provides information on tissue vascularity, oxygenation, and optical scatter.[2–4] Magnetic resonance elastography (MRE) images tissue elasticity and hardness,[5–7] which are properties based on the tissue stroma and long associated with breast malignancy as exploited in clinical breast exams. Other electromagnetic imaging methods[8,9] are believed to be sensitive to tissue cellularity, cell membrane integrity, bound water content, and ionic concentrations, which, for example, have been hypothesized to be signatures of breast malignancy.[10–15]

One conceptual approach to developing relevant pathological correlates of imaged tissue characteristics is to focus on the properties of the tissue compartments that change with disease progression such as (a) epithelial cells, (b) supporting stromal matrix, and (c) blood vessels. The operative concept is that, while the initiating event in disease pathogenesis (e.g., neoplastic transformation) may not be optically visible, the growth and progression of the disease process will become optically detectable. In the first instance of epithelial cell proliferation, alterations in cellular density are characteristic optical and histopathological signatures of cancer. Indeed, pathologists are trained to observe, compare, and contrast subtle changes in cell morphology (such as nuclear size, shape, and chromatin pattern; nuclear membrane irregularity; and nucleolar number and shape) and cellular density when making a clinically relevant diagnosis, be it benign, hyperplastic, dysplastic, or malignant. Optical techniques are now being developed to recapitulate these subcellular morphologic changes. Using scattering-based methods, point probe systems can now measure microscopic features, such as nuclear size and subcellular particle size, in sites such as the cervix and esophagus.[16–19]

In both the first and second instances (epithelial versus stromal changes), optical scattering, electrical current flow, and capacitance are dramatically influenced by changes in extra- and intracellular densities of macromolecules such as proteins and nucleic acids. Collagen is known to be mechanically stiff and, hence, elasticity is a key parameter for fibrous tissue stroma. Lastly, assessment of angiogenic activity in relation to collagen content may provide an indication of the presence of provisional versus mature stroma; the former being strongly associated with elevated water content. Water is an important contributor to electrical permittivity and conductivity as well as optical absorption and mechanical compressibility (or incompressibility). Blood strongly affects optical absorption as well as electrical conductivity and, to a lesser extent, electrical permittivity. Another approach to validating the image findings may be to use mathematical models to statistically compare the content of multiple independent microscopic biomedical images based on multinomial distribution. Such a model has already been used to demonstrate the synergistic effect of combination treatments (chemotherapy or radiation or both) on cancer.[20]

An important finding of these various studies is that the discriminating tissue findings are not necessarily the cancerous tissues. The supporting stroma, vasculature, and influx of inflammatory cells may be just as important or more so as optical "signatures" for noninvasive optical imaging.

Postulated pathology correlates can be categorized according to the properties being validated (Table 2). Physical properties in imaged tissues (hardness and compressibility) might be assessed by comparing the different tissue-type volumes (fat versus epithelium versus stroma) at both light and elec-

Table 2 Tissue correlates of optical imaging parameters.

Parameter	Analytical Exercise
Morphometry	Morphometric properties of epithelial layers
	Nuclear density Nuclear contour Nuclear texture Cytoplasmic texture
	Morphometric properties of tissue compartments
	Adipose tissue versus fibrous tissue versus vasculature versus epithelium
	Inflammation
	Vasculature: areal density, orientation
	Collagen: density, texture, composition
	Necrosis
Immunohistochemistry	Semiquantitative image analysis
	Quantitative image analysis
	Molecular analysis of immunoreactive proteins
Fluorescence	*Ex vivo* morphologic analysis
	Ex vivo fluorescence: spectroscopy, Fuorescence: spectroscopy
	Autofluorescence Applied fluorophores
	Molecular analysis of autofluorophores and applied fluorophores
Immunofluorescence	Semiquantitative image analysis
	Quantitative image analysis
	Molecular analysis of immunofluorophores
Cytomics	Spectral imaging of cellular and tissue compartments
Spectroscopy	Molecular analysis of tissue (comprehensive or selected)
	Genomics Proteomics Metabolomics Oxygenation status Redox status

Table 3 Technologic correlates of optical imaging versus pathology.

Optical Imaging Technology	Pathology Analysis
Light scattering	*Ex vivo* morphological analysis
Confocal imaging	*Ex vivo* morphological analysis
Autofluorescence	*Ex vivo* morphological analysis, spectral analysis
Exogenous fluorophores	*Ex vivo* morphological analysis, spectral analysis
Mie scattering	*Ex vivo* spectral analysis
Near infrared	*Ex vivo* spectral analysis
Raman spectroscopy	*Ex vivo* chemical analysis
Elastic scattering spectroscopy	*Ex vivo* morphological analysis

tron microscopic levels. Measures of vascularity, reflecting both angiogenesis per se and vasculogenesis in general, might include vessel density, area, orientation, and tissue hypoxia or necrosis. Protein expression, analyzed by immunohistochemistry, immunofluorescence, or serum or tissue proteomics, can define angiogenesis (stimulatory or inhibitory), tissue oxygenation, stromal components, cell-cycle regulators, cytokine production, proliferation correlates, proteolytic enzymes degrading the extracellular matrix to facilitate tumor stromal invasion, and metastasis suppressors or enhancers.[21] Autofluorescence spectroscopy in the ultraviolet-to-visible range may detect differentiating endogenous fluorophores in the epithelial and extracellular matrix.[22,23] Phase-contrast signals in frozen tissue may reflect optical scatter. Gene expression profiles can be used to define morphologic phenotypes, cellular adhesion, extracellular matrix, angiogenesis, and therapeutic targets. We may not yet have a clear understanding of which molecular alterations correlate with imaging findings (cancer initiation and progression versus cell-cycle regulatory proteins versus tumor-suppressor genes versus treatment response), but interinstitutional research groups should develop standardized ways of archiving tissue so that it is available for future collaborative gene array studies. Limitations encountered in NTROI studies to date include precision of sampling of the lesional tissue analyzed by optical methods *in vivo*;[24] the accuracy of the parametric morphologic measurements *ex vivo*; and the reliability of pathology diagnosis (to be discussed).

Perhaps most important of all, we need to ensure that standardized interinstitutional data is weighted according to its relevance in clinical outcome versus its utility in validating the imaging technology. The chosen pathology correlates must be able to differentiate a range of clinically relevant differential diagnoses: normal; benign; hyperplastic; dysplastic; malignant, noninvasive; malignant, invasive. The validation methodologies must be applied to different biopsy types depending on the organ site (biopsies, cores, excisions). Moreover, it is not a safe assumption that optical imaging or spectroscopic parameters operate on a monotonic continuum as disease progresses. The possibility must be allowed that optical signatures change in a discontinuous fashion as disease progresses. Only experimental evidence can establish whether "parametric thresholds" or optical signatures are the appropriate basis for clinical decision making. These efforts are ongoing within NTROI (Table 3).

Wherever possible, the tissue must be oriented in relation to the patient and the three-dimensional orientation of the imaging method, so that direct morphologic correlations can be obtained. The need for orientation applies to both macroscopic (gross) and microscopic analysis. This is mandatory in examination of excised surgical specimens. In the case of bi-

opsy specimens, orientation to the relevant tissue planes (e.g., mucosa and submucosa) optimizes the likelihood of meaningful correlation with the optical findings obtained prior to tissue harvest. An excellent example of such successful correlation is the recent implementation of confocal laser endomicroscopy, which utilizes the superior spatial resolution of confocal microscopy at the time of endoscopy to better understand the *in vivo* microarchitecture of the bowel. Used in conjunction with chromoendoscopy, the analysis of mucosal surface details is beginning to resemble a histologic examination. This technique has been applied to the *in vivo* diagnosis of Barrett's epithelium and associated neoplasia, intraepithelial neoplasia in ulcerative colitis, microscopic colitis, and Helicobacter pylori.[25,26] Studies have already reported excellent predictions of Barrett's esophagus (sensitivity of 98.1% and specificity of 94.1%) and associated neoplasia (sensitivity of 92.9% and specificity of 98.4%) using this technology.[27] Kappa statistics for inter- and intraobserver agreement for the prediction of the histopathological diagnosis are 0.843 and 0.892, respectively, raising the question of whether repeated screening gastrointestinal biopsies interpreted by a pathologist will be needed.[27] Whether these optical images will be interpreted at the time of endoscopy by the gastrointestinal physicians or will be sent (as images) to a pathologist for a definitive interpretation remains to be seen and raises important questions as to the future training of new medical specialists.

3 Pathology Standardization and Validation

3.1 *Diagnostic and Reporting Reproducibility*

Over the last 10 years, pathologists have defined specific diagnostic areas in different organ sites where there is poor diagnostic reproducibility between pathologists. Examples of such diagnostic pitfalls include the distinction between atypical benign proliferations versus noninvasive low-grade carcinomas in breast;[28,29] Gleason grading in prostate cancer,[30] Fuhrman grading in renal cell carcinomas;[31] and severity grading in Barrett's esophagus dysplasia.[32] Pathologists have studied and published both intra- and interobserver diagnostic variability between experts and generalists, nationally and internationally. Where possible, they have refined or simplified diagnostic classification systems to make them more reproducible and clinically relevant. National consensus committees throughout the medical world have now published guidelines and standardized reporting templates for tumor diagnoses, unique for every organ system (e.g., Ref. 33). These ensure that the same common diagnostic data elements are collected for every tumor diagnosis, with identical terminology, so that clinical prognosis and outcome data can be compared and contrasted across institutions.

For pathology-imaging correlation validation, these areas of interobserver diagnostic variability, while infrequent, must be identified and the chosen classification schemes tested among the study pathologists for diagnostic reproducibility and standardization of reporting language and nomenclature. Participating validation pathologists should be required to use standardized, nationally recognized consensus reporting templates, if they exist. In the small percentage of study cases where the pathologic diagnosis does not have optimal reproducibility, another morphologic correlate, not routinely used to make a tissue diagnosis, could be considered (such as angiogenesis, hypoxia, cytokine production, etc.).

Interobserver variability in the diagnosis of neoplasia is a key limiting factor in the ability of pathology to serve as a gold standard for optical imaging. However, despite concern about whether histopathology is a gold standard or a tin standard, a striking fact remains: that pathology is an extraordinarily valuable management tool for patient care. The rigor of histopathological interpretation of human tissues is one of the great success stories in evidence-based medicine[34] and relies upon the experience and knowledge of the practicing pathologist.[35] Although pathology interpretations are ultimately reduced to categorical data points in research studies, it is such diagnostic interpretations that constitute the practice of medicine, with all of its inherent risks and responsibilities.

The optical imaging community should be aware that management of patients on the basis of optically acquired images and data will be subject to the same rigorous standards of reproducibility, specificity, efficacy, and ultimate benefit to patient care as are currently applied to pathology. Hence, the discussion of pathology standardization in support of optical imaging research takes on added importance, because ultimately optical imaging will itself have to stand alone as a diagnostic entity. It is interesting to note that, as of October 29, 2006, PubMed [(www.pubmed.gov) a service of the National Library of Medicine and NIH] listed 4172 published papers addressing the topic of "interobserver variation and pathology," the majority pertaining to diagnosis of dysplasia and cancer.[35] The topic of "interobserver variation and optics" garnered 3001 published papers, largely pertaining to ophthalmology. The topic of "interobserver variation and optical imaging" produced only 48 published papers. One might conclude that the evaluation of interobserver variability in interpretation of optical imaging diagnostics is still in its earliest stages.

3.2 *Tissue Processing and Involvement of a Study Pathologist*

Research in optical imaging often requires delay in the delivery of tissue specimens to the pathology laboratory, owing to possible *ex vivo* optical imaging procedures that must first occur. Hence, the tissue specimens available for gold standard correlation may not be of optimal quality, but may have been degraded by dessication, heating, or mechanical deformation.

In the early planning of imaging validation studies, it is essential to contact a pathologist with appropriate diagnostic expertise in the organ under study. The first step is obtaining advice regarding tissue sampling, processing, and archiving, as well as assistance with Institutional Review Board (IRB) proposals related to human tissues. A pathologist can then help in the design and execution of optical imaging schemes, including selection of possible validation correlates. This includes teaching the optical imaging research team the morphology, pathobiology, and terminology of the site to be imaged. Indeed, terminology consensus (medical versus technical) is essential. A pathologist will ensure that the clinical standard of care for a tissue diagnosis is not compromised by the study design, especially by ensuring optimal quality of tissue delivered to the pathology laboratory so as to achieve

optical diagnostic accuracy and reproducibility for the clinical lesions being imaged.[36]

The most important priority for a study patient's specimen is to ensure that the tissue is processed in such a way that the usual diagnostic and prognostic criteria can be detailed in the pathology report, according to standard-of-care clinical reporting practices. Uniform preanalytical processing of tissue specimens is crucial for both the pathologic diagnosis and successful validation analysis. The method of tissue preservation used may depend on the organ site, biopsy method (fine-needle aspirations, cores, excisions), or tissue diagnosis. The preservation of marker probes, such as tissue antigens, fluorescence, or dyes, may be affected by tissue fixation or the time to tissue processing. The optical parameters of interest will also dictate what specific pathology analysis will be required (Table 3). Lastly, a pathologist is critical for assisting in the interpretation of optical findings. This is best accomplished by educating the study pathologist, in turn, about the optical technologies being deployed. We assure the optical imaging community that such education is a very rewarding part of participation in the research program.

According to inspection bodies such as the College of American Pathologists (CAP) and the Royal College of Pathologists (United Kingdom), the current clinical standard-of-care processing method for biopsy tissue is formalin fixation, dehydration through graded alcohols, tissue impregnation or embedding in paraffin wax, tissue sectioning, and staining with hematoxylin and eosin (H&E). Wherever possible, tissue processed in this way should be used in early validation studies for the following reasons: (1) the gold-standard clinical diagnosis is preserved; (2) there may be insufficient lesional tissue (in excess of that required to generate a complete pathology report) to process in alternative ways such as fresh-frozen tissue sections, snap-frozen tissue banking, or glutaraldehyde fixation for electron microscopic analysis; (3) all published diagnostic accuracy and reproducibility studies reflect tissue processed in this manner; (4) there will be abundant, preserved, archived tissue for future correlating studies.

Historic limitations in validating possible pathology correlates include the specimen processing, type, and orientation. In 1990, with the advent of affordable and accessible image processing technologies, the Committee for Diagnostic Quantitative Pathology Working Group of the European Society of Pathology called for the widespread standardization of preparatory tissue techniques, interpretational criteria, and internal quality assurance. Variations in the time taken to process tissue, its fixation method, dehydration, paraffin embedding, water-bath temperature, and sectioning thickness all influence correlate measures performed on routinely stained sections.[37–39] In addition, immunohistochemical techniques also vary.[40] Wherever possible, the validating laboratories participating in a multiinstitutional study must use standardized methods and reagents with automated tissue processing and staining technology.

3.3 *Quantitative Analysis and Measurement Method Reproducibility*

Semiquantitative evaluations in pathology, such as severity of disease (mild, moderate, severe) or degrees of immunohistochemical staining (scores such as 0, 1+, 2+, 3+, 4+) can be subjective and particularly difficult to reproduce. Semiquantitative evaluations of diagnostic criteria depend on the pathologist's experience, the number and complexity of the tiers in the classification scheme, and the tissue sectioning or staining quality. Studies have shown that the smaller the number of options in a semiquantitative classification scheme, the better the reproducibility, which is best achieved if there are only two options (present or absent).[41,42]

Wherever possible, semiquantitative measures should be replaced by quantitative analysis using computer-assisted image processing instrumentation.[43–45] Automated computer-assisted quantitative image processing techniques are essential because of the ever-expanding list of biomarker surrogates under evaluation and development that can be rapidly sampled through tissue and gene arrays. Automated acquisition of composite, high-resolution digitized tissue section images with rapid, precise image analysis is also needed to ensure standardized objective assessments. Whether the image processing system has a commercially available or a custom computer platform, method validation and laboratory intra- and interobserver reproducibility must be documented between collaborating institutions so that validation data can be compared across research networks.

3.4 *Technical Reproducibility and Reporting by Biomedical Scientists*

In the same way that pathologists have been forced to acknowledge, refine, and standardize tissue processing methodologies and diagnostic reproducibility, the optical imaging community should also consider: (1) universal standards for instrument standardization; (2) intra- and interobserver image or biophysical signature interpretation variability; (3) measurement repeatability; and (4) technical reporting of specification details (e.g., optical filters, photocathode sensitivity, dynamic ranges).

4 Quantitative Reference Standards and Data Sharing

Currently, quantitative reference standards are often derived from single studies of small sample size. There is an urgent need to share standardized data across institutional research groups rather than developing local standards. The NCI-funded CaBIG (Cancer Biomedical Informatics Grid) project is creating the tools and awareness for how intra- and inter-institutional systems can communicate to share medical data, but their success depends on universal access to standardized common data elements of information such as the pathology details, tissue availability, laboratory tests, and clinical outcome data.[46] The exchange of a wide variety of data on the Web can be achieved using simple, flexible text-format-derived languages such as the Extensible Markup Language (XML). For example, this would enable universal access to the digitized images of multiple paraffin-embedded tissue samples in tissue microarrays (TMAs) with the central database providing both patient clinical information with outcomes and molecular analytical data (immunohistochemistry, *in situ* hybridization, etc.).[47]

The ideals of standardized data exchange are shared by the Pathology Working Group within the NCI-funded NTROI, and the Laboratory Digital Imaging Project (LDIP) of the

Table 4 Ongoing tasks for pathology support of optical imaging.

Technical Operation	Parametric Analysis
Physical performance of the instrumentation	Signal to noise Reliability Reproducibility Precision Efficacy
Instrument interaction with examined tissue	Mechanical deformation Optical alteration Dessication
Limits of resolution	Morphologic Spectroscopic
Instrument processing of data	In-line data processing Artificial intelligence
Biomarkers	Molecular design Molecular verification Access to tissue compartments Signal-to-background and detection
Quantification of tissue parametrics	Digital morphometry Spectroscopy
Data management	Archiving Standardization Annotation Integration

NIST. NIST is currently working with CAP to develop standardized check samples for Her2/Neu well as standards for verifying performance of optical imaging devices. The ability to image and quantitate fluorescently labeled markers *in vivo* has generally been limited by the autofluorescence of formalin-fixed tissue specimens. Now, with the advent of increased capabilities in existing fluorescence and brightfield microscopes, fast, accurate, and affordable multispectral analysis can be obtained. As *in vivo* imaging technology evolves, the need to validate *in vivo* images with pathology correlates will further increase. These efforts are ongoing (Table 4).

5 Recruitment of the Pathology Community

A final activity of the Pathology Working Group is to publicize to the academic pathology community the importance of expanding the technologies practiced in pathology, especially those relevant to optical imaging. Accordingly, efforts involving automated analysis of cytometric images[48] and fast-Fourier analysis of nuclear morphometry[49] and collagen structure[50] are to be commended. In each instance, the sensorium of pathology is being expanded and in ways that may be relevant to the practice of optical imaging. Efforts to map histologic tissue sections to optical images[51] also will be of value. This includes using tissue autofluorescence of NADH and flavoproteins to assess myocardial apoptosis,[52] bioluminescence measurement of cancer to predict radiosensivity of individual malignancies,[53] and uptake of fluorescent biomarkers to predict tumor aggressiveness.[54] Moreover, a shift of pathology immunodiagnostics from immunohistochemistry to rigorous immunofluorescence[55] may have direct relevance to the field of optical imaging. Regardless of modality, integration of molecular diagnostics into the routine algorithms of diagnostic pathology may routinely require rigorous quantitative approaches to immunodiagnostics.[56–59]

6 Summary

The validation of an image signal with a biological basis for image contrast, using biomarkers derived from pathology gold standards, is essential for the adoption of novel imaging techniques into medical practice. For a predictive or prognostic disease biomarker to be useful, it must be clinically important, independent, significant, and standardized with regard to methods, interpretation, and data reporting.[60] The same must apply to image-correlating biomarkers. Rigorous validation of such biomarkers must be performed with the goal of a standardized, reproducible, efficient format for clinical diagnostic implementation.[61] The pathologist, by providing diagnostic reproducibility, access to correlating biopsy material, and molecular expertise, should be an integral part of any team that is validating novel imaging modalities.

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