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DETECTION OF EARLY CANCEROUS CHANGES AND CANCER IN BLADDER TISSUE BY AUTOFLUORESCENCE AND REFLECTANCE

Volume I

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy in the Graduate

School of The Ohio State University

By

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***

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1999

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Graduate Program in
Biomedical Engineering
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1999
Most new bladder cancers are transitional cell carcinoma (TCC). Dysplasia, which can progress to TCC, and a flat low-grade TCC known as carcinoma-in-situ (CIS) can progress into invasive TCC. Visual identification through a cystoscope is unreliable and taking random biopsies for a more reliable diagnosis is expensive. Differences in the observed fluorescence or reflectance spectra collected using an optical fiber probe could lead to guidance for taking fewer, better chosen biopsies or a diagnosis from the observed spectra alone. Two studies explored this: an in vivo study investigated papillary TCC and an in vitro study investigated dysplasia, CIS, invasive TCC, and papillary tumors. The spectra were analyzed with a multivariate technique of principal component analysis with logistic regression (PCA/LR). In all cases, biopsies were taken and evaluated by a pathologist.

For the in vivo study, at 400 nm excitation, which performed better than 370 nm, 23 non-diseased and 22 diseased sites from 10 patients analyzed with cross-validation to blind the PCA/LR algorithm achieved an 82% sensitivity and 96% specificity. Results without validation are often reported in various articles and a 100% sensitivity and 100% specificity was achieved without validation. This was detecting papillary TCC.
For the *in vitro* study, 400 nm excited fluorescence performed best, although reflectance was not much worse suggesting absorptive effects are important. At 400 nm excitation with dysplasia and CIS classified as diseased, 84 non-diseased and 20 dysplasia and 25 sites of CIS from a composite of 30 patients achieved only 60% sensitivity and 66% specificity even without using a validation set. In contrast, papillary TCC and invasive cancer were detected well at both excitations and with white light reflectance.

Quantitative fluorescence microscopy of frozen unstained bladder sections identified the fluorophores as cross-linked collagen and occasional inflammatory cells, with the same fluorophores at 380 or 400 nm excitation. Bladder epithelium did not display fluorescence regardless of being benign, dysplastic, or CIS, which explains the difficult of differentiating dysplasia and CIS from benign mucosal tissue.
Dedicated to my parents
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PUBLICATIONS

1. G.I. Zonios, R.M. Cothren, J.T. Arendt, J. Wu, J. Van Dam, J.M. Crawford, R.
Manoharan, M.S. Feld, "Morphological Model of Colon Tissue Fluorescence," IEEE

2. R. Manoharan, G. Zonios, R. Cothren, J. Arendt, J. Van Dam, M.S. Feld, "Laser-
induced Fluorescence Spectroscopy: Optical Histological Analysis of Colonic Dysplasia,"

3. J. Zilberberg, B.L. Davis, G.M. Wildey, C.A. McDevitt, J.T. Arendt, and P.R.
Cavanah. Diabetic Complications Conference, A Joint Symposium in Celebration of the
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**FIELDS OF STUDY**

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CHAPTER 1

INTRODUCTION AND OVERVIEW

1.1 Overall Objectives

The purpose of this thesis is to measure and characterize the intrinsic steady state fluorescence achieved without dyes (autofluorescence) and diffuse reflectance of the urinary bladder for the purpose of finding various stages of transitional cell carcinoma (TCC).

A preliminary study with fluorescence microscopy of colon tissue attempted to explain previously measured and published in vivo colon spectra. [1, 2] For this thesis, three studies investigated bladder autofluorescence: an in vivo cystoscopy study, an in vitro cystectomy study which examined the bladder within half an hour of removal from the patient, and a fluorescence microscopy study of frozen bladder tissue biopsies.

Unlike biopsies which require the removal of tissue, autofluorescence and reflectance spectral measurements could be performed in vivo without tissue removal and without staining, yet reveal critical morphologic and chemical information. For the spectroscopy studies in this thesis, some spectra were indeed taken in vivo, but there was also an in vitro bladder study preformed after cystectomy, which removes the bladder
from the body, within thirty minutes after its removal. Because of the available patient population, use of the extracted bladders allowed greatly increasing the sample size and observation of a greater variety of forms of TCC.

Excitation light directed at the tissue surface induced fluorescence emission at wavelengths characteristic of its structural and molecular makeup. The resulting fluorescence spectra might distinguish benign tissue from TCC if the tissue types have different chemical compositions or morphology. Since this technique can provide real-time assessment, it will potentially reduce the sampling error associated with biopsies taken at cystoscopy and allow additional regions of normal-appearing bladder wall to be screened for premalignant lesions.

For the fluorescence microscopy study, frozen unstained sections were imaged on a fluorescence microscope to identify and reveal the morphology and location of the fluorescing structures (fluorophores) within the tissue.

1.2 Fluorophores and Their Morphology in Tissue

In biological tissue, the complex and broad fluorescence spectra result in difficulties in determining the exact chemical composition of the fluorophores. [3] As a result, much of the use of autofluorescence to diagnosis precancerous changes in tissue has not put much emphasis on the exact nature of the fluorophores and absorbers, but more often merely speculated on these while concentrating more on whether the spectra themselves provide diagnostic information. For example, many studies report that autofluorescence can detect dysplasia in the colon. [4, 5, 6, 7] While these studies
demonstrate the usefulness of autofluorescence in detecting disease, they lack a solid, quantitative analysis of why this fluorescence occurs, instead giving empirical explanations. Suggested fluorophores responsible for the fluorescence observed in colon tissue include elastin, collagen, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NADH), phenylalanine, pyridoxal 5’ phosphate, tryptophan, and tyrosine. [7, 8, 9, 10, 11] With excitation varied from 240 nm to 340 nm, Banerjee et al. found the three most likely fluorophores responsible for the colon spectral response to be tryptophan, collagen type IV, and perhaps NADH with hemoglobin causing absorption dips while elastin, FAD, phenylalanine, pyridoxal 5’ phosphate, and tyrosine did not seem to be obviously contributing to observed emission peaks. [9] Banerjee et al. also noted multiple emission peaks for collagen IV dependent on the excitation used so that with excitation at 310-350 nm, the emission peak is at 365 nm while excitation at 330-380 nm creates an emission peak at 430 nm. [9] Thus, it may be important to consider both excitation and emission for determining potential fluorophores as the emission peak may not remain stationary.

Out in the red area of the emission spectra, Yuanlong et al. noted that cancerous regions from stomach, esophagus, tongue, mandible, and bladder tissue had peaks at 630 nm and 690 nm which were most likely caused by endogenous porphyrins. [12]

To better understand the autofluorescence from tissue, knowledge of cell types and morphology is required as well as knowing the chemical composition.

In the preliminary study for this thesis with fluorescence microscopy of colon tissue at 380 nm excitation, it was found the collagen in colon tissue fluoresces which
was expected. More revealing was that another fluorophore was the cytoplasm of
dysplastic crypt cells without detectable fluorescence form the cytoplasm of normal crypt
cells. Yet another fluorophore was found to be round yellowish-green spots assumed to
be inflammatory cells.

Even when only considering collagen as a fluorophore since it was the strongest,
morphology including the thickness of the lamina propria with its lesser amount of
collagen compared to the greater and more densely packed amount in the deeper
submucosa also provided important diagnostic information. This morphology is certain
to effect measured fluorescence spectra from the tissue surface. [2]

Koenig et al. investigated autofluorescence of bladder tissue at other excitation
wavelengths than the studies for this thesis, but still relevant is their theory that
morphological changes as well as the type of fluorophores contribute to the emission
fluorescence spectral response of benign bladder tissue and papillary tumors. [13] A
thickened epithelium in papillary tumors causes the lamina propria to be further from the
tissue surface. This results in less excitation light reaching the lamina propria to cause
fluorescence and less fluorescence emission light getting back to the tissue surface.
Therefore, for the fluorescence emission spectra collected at the tissue surface, a smaller
portion will be contributed from fluorescence in the lamina propria and more from the
epithelium itself compared to the spectra collected from benign tissue. Thus,
Koenig et al. are considering two layers in the fluorescence model, the epithelium (also called urothelium) and the submucosa with these influencing the returned fluorescence spectra. [13]

Richards-Kortum attributes the emission fluorescence in the red part of the spectra that seems to be from porphyrins to porphyrins in bacteria. She cites an earlier study that found a lack of red fluorescence in culture for ulcerated squamous cell carcinoma. [14] Our study investigated transitional cell carcinoma so it is unclear if the results for squamous cell carcinoma will also apply. Dr. Howard Levin, the pathologist who reviewed the microscope slides for this thesis, noted he didn't see bacteria in the hematoxylin-and-eosin (H&E) stained slides and would have expected to if they had been present.

Recent studies show that fluorescence spectroscopy can detect adenomatous transformation of the colon [1, 4, 7, 8, 10, 11, 15, 16, 17, 18, 19, 20], squamous cell carcinoma and adenocarcinoma in the esophagus [20, 21, 22], dysplastic epithelium and carcinoma of the lung [23, 24, 25], cervical intraepithelial neoplasia [26, 27, 28], atherosclerotic lesions [29, 30], papillary tumors in the bladder [16, 31, 32, 33, 34, 35, 36], and other malignant tumors in the bladder [13, 33, 36, 37, 38, 39, 40]. One intriguing bladder autofluorescence study concentrated on the crosslinking of collagen in the bladder in rats with induced diabetes, which illustrates it is the crosslinking rather than collagen itself producing the fluorescence. [41]
1.3 Objectives Specific to Bladder Tissue

Most new bladder cancers are transitional cell carcinoma (TCC) and low-grade TCC can progress into invasive TCC. [42] Cystoscopic visual examination and random biopsies, which are limited in number because they remove tissue, can easily miss morphologically flat carcinoma-in-situ (CIS). [43] This is a serious concern because CIS is a likely precursor to developing invasive tumors. [43] However, differences in the observed fluorescence and reflectance spectra collected without tissue removal using an optical fiber probe performed during cystoscopic examination can lead to guidance for taking fewer, better chosen biopsies or a diagnosis directly from the observed spectra. Two studies in this work explore this: an in vivo study investigating papillary TCC and an in vitro study investigating flat TCC.

Currently, superficial bladder tumors are observed in only a minority of patients prior to a diagnosis of deep invasive tumor. [44] However, the correlation between the easily observed superficial bladder tumors called papillary tumors, which stand up from the mucosal surface like a tree, and subsequent invasive cancer is low. The morphologically flat CIS is much harder to detect, yet is believed to be a link in the chain leading to invasive bladder cancer. [45] In the United States, bladder tumors that invade into the muscularis mucosa most often result in a radical cystectomy, which is removal of the entire bladder, with a 10% failure rate. [44] England and Canada more commonly use a full course of radiation therapy, with a 50% failure rate, and only follow that three to six months later with a cystectomy if unsuccessful, for an overall failure rate not much different than that in the U.S. [44]
One goal of this research is easily and reliably detecting dysplasia and CIS in the bladder. While CIS may eventually progress into a lethal tumor, it is at an early, superficial, localized stage which makes it more easily treatable. [45] Perhaps with treatment at the dysplastic or CIS stage rather than when already progressed to an invasive tumor, fewer cystectomies would need to be performed.

1.4 How Objectives Differ From Previous Work in the Bladder

1.4.1 Bladder Fluorescence At or Near 370 nm and 400 nm Excitation

Reports on bladder autofluorescence exist in the literature. [13, 16, 29, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40] For our in vivo and in vitro bladder autofluorescence studies, excitation wavelengths of 370 nm and 400 nm were chosen. This matches fairly closely the excitation maximums found by Audin for a variety of mammalian cells. Audin credited the excitation peak at 360 nm with an emission peak at 460 nm in the cells to NADH. Audin credited the excitation peak at 400 nm with an emission peak at 520 nm to riboflavin although riboflavin itself in phosphate-buffered solution (PBS) has an excitation peak at 460 nm, with a broad range though, with an emission peak still at 520 nm. [5] Since the mammalian cells fluoresced well at these excitations, this suggested these two excitations might also work well for causing fluorescence in bladder epithelial cells.

D'Hallewin et al. successfully used fluorescence spectroscopy to detect bladder TCC and CIS without the use of exogenous dyes at excitations of 355 and 365 nm. [31, 32, 34, 35] In one study, 355 nm excitation light was used. [31, 32] The emission
spectra had a broad peak centered at 455 nm which slowly decayed at longer
wavelengths. Papillary TCC was found to produce less fluorescence by a factor of 2.7,
and CIS by a factor of 2.2, as compared to benign bladder mucosa. [31, 32] Using seventy
percent of the average benign mucosal peak height as a threshold for determining the
diagnoses, the results were remarkably good. Two out of 235 benign mucosal tissue were
incorrectly diagnosed as diseased, 2 out of 173 of the papillary tumors were incorrectly
diagnosed as diseased, and 1 out of 19 CIS were incorrectly diagnosed as diseased.
Combining papillary tumors and CIS into one diseased category, that is a sensitivity of
98% and a specificity of 99%. [31]

When D'Hallewin et al. used 365 nm excitation light, rather than collecting
spectra with a spectrometer, the emission light collected after passing through a 50 nm
bandpass filter centered at 450 nm was used as the sole diagnostic criterion. [34]
Papillary TCC was found to produce less fluorescence by a factor of 3.2, and CIS by a
factor of 2.6, as compared to benign bladder mucosa. [34] Again using seventy percent of
the average signal for benign mucosal tissue as a threshold, this time all the benign
mucosal tissue was correctly diagnosed as non-diseased and all papillary tumors and all
four of the CIS sites were correctly diagnosed as diseased. This was with sixteen
patients with an unspecified number of sites taken in each patient. However many sites
there were, since nothing was misdiagnosed, this achieves 100% sensitivity and 100%
specificity for detecting papillary tumors and for detecting CIS. [34]

D'Hallewin et al. attributed the fluorescence to both collagen and NADH. At
365 nm excitation, they found collagen to have an emission peak at 415 nm and NADH
to have a peak at 455 nm. However, they also noted that hemoglobin strongly absorbs at 415 nm too. These two fluorophores and the one absorber are what they attribute the fluorescence results to. [34]

D'Hallewin's extremely promising results at 365 nm excitation are worthy of further investigation, as well as trying the 400 nm excitation which Audin noted was an excitation maximum for mammalian cells.

Baert et al. used 405 nm excitation, which is close to the 400 nm excitation used in this study, but their study used Photofrin dye, a hematoporphyrin derivative, to cause additional red fluorescence where preferentially absorbed by fast growing tumors. Baert et al. found a peak at about 460 to 480 nm for benign mucosal tissue but it appears from their graph that this peak is down by at least a factor of ten for papillary TCC. They attributed this peak solely to autofluorescence since the Photofrin dye should only add fluorescence in the red part of the emission spectrum. For the average tumor graph, the peaks at 630 nm and at 680 nm resulting from the Photofrin dwarf its peaks around 450-500 nm. [46] The emission from 600-750 nm will be affected by Photofrin making it hazardous to compare those results to our autofluorescence study. At shorter emission wavelengths, somewhat similar results were expected and, as will be shown later, observed.

Jichlinski et al. used topical administration of 5-aminolevulinic acid which causes an increase in intracellular protoporphyrin-IX (PpIX) accumulation, with PpIX a fluorophore. They achieved 82.9% sensitivity and 81.3% specificity with a broad excitation from 380-450 nm. [47] A 490 nm long-pass filter removed the blue
wavelengths and the 460 nm peak that Baert attributes to autofluorescence in spite of the dye he uses. [46] The spots that had the PpIX fluorescence appeared red whereas the bladder wall appeared green. It seems the fluorescence spots were visualized with a cystoscope for their study rather than measured spectroscopically. [47] This combined both 41 flat tumors (including dysplasia and CIS) and 6 papillary tumors into one diseased category and 91 benign mucosal tissue sites as the non-diseased category. However, this excludes the papillary tumors visible to the cystoscope. It seems 96% of those were detected. [47] This does not seem to translate well to the autofluorescence examined in our study.

Eika et al. did a study solely on the removed collagen from bladders in a study investigating collagen cross-linking in rats with induced diabetes. The bladder samples taken from the rats were lyophilized, delipidated, and hydrolyzed. With 370 nm excitation, the emission at 451 nm was taken which is close to the yellow emission peak. [41] This experiment is of particular note in relation to our studies because it extracts and measures one fluorophore.

Both the in vivo and in vitro fluorescence studies for this thesis include both 370 nm and 400 nm excitation taken consecutively on the same samples within only a few minutes of each other. It will determine which of these is the preferable excitation for diagnosing TCC in bladder tissue with autofluorescence spectroscopy.

Unlike the techniques in the bladder fluorescence articles listed so far which extracted only certain emission values for processing in their models, for this thesis the spectra were processed using principal component analysis (PCA) and logistic regression.
(LR), as will be discussed in a later chapter. Important to note for the objectives of this thesis at this point is that using PCA and LR allows use of the entire spectra without a priori selection only certain wavelengths and potentially losing valuable information.

1.4.2 Bladder Fluorescence at Other Excitation Wavelengths

Koenig et al. preformed autofluorescence with excitation at 337 nm. [13, 36, 39] Their algorithm uses the ratio of the emission intensity at 385 nm to 455 nm. [13, 39] This algorithm cannot be tested on the experiments for this thesis because when an excitation of 370 nm as we used, a 400 nm long pass blocking filter was used. Thus, if there was a 385 nm emission signal was not measured.

Koenig et al. reported a sensitivity of 97% and a specificity of 98%. [39] With results that good, it might seem 337 nm excitation is the best excitation wavelength to use. However, this was not a prospective study. Instead, the data was collected as one large set and then a threshold was decided upon. A later study by Koenig et al. excluded obvious papillary lesions and the decision threshold was changed from 1.01 to 1.78. [13, 39] The later study had more CIS and that caused the higher decision threshold with the diagnosis of benign if above the threshold and TCC if below it. This is an indication CIS will likely be harder to diagnose than papillary tumors if this trend continues at 370 nm and 400 nm excitation. For the later study by Koenig et al., sensitivity and specificity are
not shown, but from an included graph appears to have a specificity when the threshold is at 1.78 considerably lower than 98% as it seems at least 13 of 107 nonmalignant sites are below 1.78, but the graph doesn't make clear exactly how many. [13]

Koenig et al. attribute their observed autofluorescence emission peak at 455 nm to NADH. [36, 39] They indicated NADH is primarily located in muscle cells but will also be located in the lamina propria. [39] The emission peak they see at 385 nm they attribute to collagen. [39]

Baert et al. had a study mentions above in the section on excitation wavelengths the same or similar to those used in this study. That same article also uses 337 nm excitation. This is a study which used Photofrin dye, a hematoporphyrin derivative, so caution is needed in assuming the results will still apply for autofluorescence. Baert et al. attribute the blue and shorter wavelength peaks only to autofluorescence despite the dye. With 337 nm excitation, benign mucosal tissue had its main emission peak at about 390 nm and a secondary peak at 460 nm while papillary tumors had its main emission peaks at 460 nm and two small secondary peaks at 630 nm and 680 nm. [46] The dual emission peaks at 390 nm and 460 nm match fairly well the two peaks used in the studies by Koenig et al. of 385 nm and 455 nm. [13, 39]

In two studies, Andijar et al. use the three excitation wavelengths of 308 nm, 337 nm, and 480 nm. [33, 37, 38] The 337 nm excitation was the closest to the excitation used in the studies for this thesis. As noted earlier, at 337 nm excitation, Koenig et al. reported dual emission peaks, one at 385 nm and the other at 455 nm. [13, 39] At 337 nm excitation, Andijar et al. only report an approximately 450 nm emission peak and not a
second emission peak, but their instrumentation includes a 400 nm long-pass filter used with 337 nm excitation which would block the 385 nm emission peak from the detector. [33, 37, 38] When analyzed by a t-test rather than a diagnostic algorithm, changes in the absolute intensity measurements of the 450 nm emission peak between benign mucosal tissue and bladder tumors, which includes both papillary tumors and invasive tumors lumped together, had a p-value under 0.05. [33, 38] No differences in spectral shape were noted in their article. Andijar et al. attributed their results at 337 nm excitation to NADH since it excites well and has an emission peak at 450 nm. [33, 37, 38]

Andijar et al.’s most strikingly positive results were at 308 nm excitation. This caused dual emission peaks at 380 nm and 440 nm. [37, 38] These peaks are only slightly shifted from the two emission peaks at 385 nm and 455 nm Koenig et al. reported, although that was at 337 nm excitation rather than 308 nm. [13, 39] Andijar et al. used a ratio of the 380 nm to the 440 nm emission peaks and a threshold of 2.0 to diagnose tumors (including papillary tumors, CIS, and invasive tumors) from benign mucosal tissue (can include inflammation) with 100% sensitivity and 100% specificity. They state the likely fluorophores are NADH for the 440 nm peak and a combination of tryptophan and collagen for the 380 nm peak, with hemoglobin absorption creating a noticeable dip around 420 nm. [37, 38] Of note is they also speculate that the lower collagen fluorescence for CIS comes from increased cell layers in the urothelium and/or larger sized cells. [37, 38]

At 480 nm excitation, Andijar et al. found changes in absolute intensity measurements for benign mucosal tissue and bladder tumors, which includes both
papillary tumors and invasive tumors lumped together, when analyzed by a t-test and using a p-value under 0.05 rather than a diagnostic test. [38] Also noted, though, was much weaker signals with comparatively greater background noise compared to their results at 308 nm and 337 nm excitation. As before, no differences in spectral shape were noted. [38]

At these same three excitation wavelengths, Andijar et al. also performed tests on cell cultures. Specifically, on normal fetal bladder labeled FHS 738 BL and human bladder TCC cell line T24 from American Type Culture Collection. [37] For the FHS 738 BL, with 308 nm excitation, they got an emission peak around 440 nm with an intensity in arbitrary units of about 2500; with 337 nm excitation, an emission peak at about 440 nm with an intensity in arbitrary units of about 200; with 480 nm excitation, an emission peak at about 520 nm, the peak location perhaps determined mainly by the cut-off filter used, with an intensity in arbitrary units of about 300. [37] The graph of the fluorescence spectra has almost no noticeable noise at 308 nm excitation, but has noise of about 30 nm amplitude at 337 nm excitation and also about 30 nm amplitude at 480 nm excitation. [37] This indicates that the urothelium of their cell culture does fluoresce, but strongly at 308 nm excitation and weakly at 337 nm and 480 nm excitation.

Andijar et al. tested the autofluorescence of individual bladder urothelial cells at excited at 488 nm. [48] As in the other article, there were also FHS738 cells for benign tissue and T24 for tumor, but also another tumor type labeled J82. They found an emission peak at about 555 nm. The intensity dropped by a factor of about ten from the spectra taken from benign tissue cells to either of the tumor types. The peak location did
not change appreciably, but the half-width of the peak was greater by up to 40% for the
tumor cells than the benign tissue cells. This work attributes the main fluorescence to
flavoproteins, with a second unidentified fluorophore contributing to the greater half-
width for tumor cells. [48]

Alfano et al. tested cancerous rat and mouse bladder tissues at 488 nm excitation
and found four emission peaks at 519 nm, 554 nm, 590 nm, and 634 nm for the rat
bladder tumor spectrum and emission peaks at 521 nm and 600 nm for the mouse
bladder. The emission peak around 520 nm is attributed to flavins and the emission peaks
between 597-634 nm are attributed to porphyrins. [40] Benson et al. found for a wide
variety of cell types, 488 nm excitation causes yellow-green autofluorescence, from 500-
600 nm, which seems the result of endogenous flavins. [61]

Some of the same suspected fluorophores, such as porphyrins and flavins, may
still excite at the 370 and 400 nm excitations used in the studies for this thesis.

1.4.3 Reflectance

Rather than fluorescence, reflectance measurements have been proposed as a
method for detecting cancer. [49, 50, 51, 52]

Zhengfang et al. processed experimentally measured colon reflectance with
multiple linear regression analysis (MLR). This differentiated adenomatous from
hyperplastic tissue with a sensitivity of 86% and a specificity of 72%. For distinguishing
neoplastic from nonneoplastic tissue, their technique yielded a 91% sensitivity and a 78%
specificity. [53] As an alternative to MLR, they also used linear discriminant analysis
(LDA) which distinguished adenomatous from hyperplastic polyps with a sensitivity of 91% and a specificity of 50% and distinguished neoplastic from nonneoplastic tissue with a sensitivity of 91% and a specificity of 74%. [53]

Mourant et al. used reflectance to find malignant vs. nonmalignant bladder tissue with 100% sensitivity and 97% specificity. This was for 10 patients with 30 nonmalignant sites and 20 malignant sites, with 19 of the twenty papillary tumors and with one CIS. [49] Separate illumination and collection fibers were used. Their theory is that the nuclear and subnuclear structures in the cells cause the most scattering. [49]

Koenig et al. used reflectance to find malignant vs. nonmalignant bladder tissue with 91% sensitivity and 60% specificity. Unlike the Mourant et al. study which focused on the reflectance at 330 and 370 nm, this study collected the reflectance spectra from 450 to 700 nm. Only the total amount of blood was found to be a useful parameter for determining malignant and nonmalignant areas, but inflammatory areas also showed increase hemoglobin in the tissue. [50]

Along with the fluorescence measurements taken at 370 and 400 nm excitation, most of the sites also had reflectance spectra taken for most of the in vitro sites measured for this thesis. As were the fluorescence spectra, the reflectance spectra were processed with PCA and LR, using the entire spectra rather than only a few extracted wavelengths.
1.4.4 Fluorescence Microscopy

Quantitative fluorescence image analysis (QFIA) has been done for cytology, which involves washings of cells out of the bladder. This was fluorescence with the dyes acridine orange and Hoechst 33258 to stain the DNA of single cells, not autofluorescence, so is not much related to the autofluorescence studied for this thesis. [54]

For colon tissue on a microscope slide at 360-370 nm excitation, fluorescence microscopy revealed blue fibers fluoresce in the submucosa and surrounding the crypts and also that the cytoplasm of dysplastic crypt cells fluoresce while the cytoplasm of normal crypt cells did not display noticeable fluorescence. [2, 55] With excitation at 488 nm, it is also found for colon tissue that the epithelial cells are minimally fluorescence while for adenomatous polyps, the fluorescence is predominantly seen in the epithelium and the lamina propria has comparatively little fluorescence. [56] For the preliminary study on autofluorescence microscopy of colon tissue for this thesis, 380 nm excitation was used and a CCD camera was used to quantitatively measure the fluorescence within the images. [2] The importance of a CCD camera instead of regular color photographic film is discussed in a later chapter on equipment.

For bladder tissue, quantitative autofluorescence microscopy was performed at both 380 nm and 400 nm excitations.

1.4.5 Calibration and Prediction Sets

Almost all of the studies, with the exception of a study by Cothren et al., used the entire set of experimental data in one large set analyzed at one time. [1] This can produce

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explained variation, but since it is not blinded does not tell the predictive value of the model. [57] For calculate sensitivity and specificity useful for finding the diagnostic potential, which is fundamentally different, the model must be tested on data not used to create the model. [57]

This differentiates the results for the studies in this thesis from most of the rest of the autofluorescence literature.

For the in vivo study for this thesis, only eleven patients had usable data at 370 nm excitation and ten patients at 400 nm excitation. A cross-validation technique was used where the algorithm was created on all the patients but one and the algorithm tested on that patient. Then, round-robin fashion, another patient was taken out and the other put back in so the test could be performed again. By doing this, the results were still blind to the data used to calibrate the model. [57, 58, 59, 60]

1.5 Achievements

Many things were learned and some initial assumptions were found to be incorrect during the progress of this thesis.

The bladder diseases examined in this thesis did not easily separate into only two categories as had been originally expected. Dysplasia and carcinoma-in-situ (CIS) were flat and to the naked eye often indistinguishable from benign mucosal tissue, while invasive cancer and papillary tumors usually had gross structural features that made it visible to the naked eye. Five categories were chosen: benign mucosal tissue, dysplasia, CIS, invasive cancer, and papillary tumors.
Initially, only fluorescence was assumed to be important for determining the fluorescence spectra with the absorption effects comparatively minor. However, the in vitro bladder tissue reflectance measurements demonstrated strong absorption effects from hemoglobin and edema. The in vitro bladder benign mucosal tissue fluorescence spectra had much greater variation when the hemoglobin and edema absorption were accounted for than when the benign mucosal tissue spectra itself were compared to the different disease categories.

Using a validation set with data hidden when developing the diagnostic algorithm with a modeling set produced significantly worse results for sensitivity and specificity especially when compared to the reported results of other researchers that did not do this, but the validation set results were more meaningful for demonstrating diagnostic potential. On the other hand, if the algorithm is developed on one set of data and tested on the same data yet produces a low sensitivity and specificity, then continuing on to use an independent and blinded validation set from that set used to develop the algorithm is highly unlikely to be profitable since the sensitivity and specificity will almost certainly get worse.

A diagnostic algorithm based on principal component analysis followed by logistic regression (PCA/LR) had the potential to improve the results since it used the full spectra rather than using only a few previously chosen and extracted wavelengths. PCA/LR did work as well or better then the other tested algorithms.
Excellent diagnoses of papillary tumors from benign mucosal tissue was found for the *in vivo* bladder fluorescence and reflectance, while the other diseases type were rarely seen in the *in vivo* study due to patient selection criteria. In contrast, papillary tumors were rarely seen in comparison to the other disease types in the bladder *in vitro* study. Good diagnoses of papillary tumors and of invasive cancer from benign mucosal tissue was found for fluorescence and reflectance for the *in vitro* study, but dysplasia and CIS were not well diagnosed.

It was found that while t-tests with p-values under 0.05 percent showed 460 nm peak heights at 400 nm excitation were significantly different between benign mucosal tissue and CIS, that did not and does not guarantee a high sensitivity and specificity when a diagnostic algorithm was made solely based on that 460 nm peak.

Bladder epithelium did not fluorescence under microscope at 370 or 400 nm excitation, regardless of whether the epithelium was benign, dysplastic, or carcinoma-in-situ (CIS).

The primary fluorophore in bladder tissue is collagen with morphology and absorption accounting for many of effects on measured spectra at the tissue surface.

Secondary fluorophores in bladder tissue were not consistently present often enough for diagnosing the chosen disease categories. One type of secondary fluorophore occasionally observed was inflammatory cells, but these only rarely appeared in the microscope images of any diagnosed tissue types. The second type of secondary fluorophore seemed to be porphyrins. Yet, the strong porphyrin peaks with
red fluorescence were seen in only a small proportion of the spectra taken and not seen at all in the microscope images. This suggests the porphyrin appearance was only sporadic regardless of the disease type. However, for the few measured spectra that did have porphyrin peaks, it seemed related to the tissue with the worst disease such as invasive cancer.
REFERENCES


CHAPTER 2

OPTICAL PROPERTIES RELATED TO TISSUE SPECTROSCOPY

2.1 Molecular Energy Changes

The energy of molecules can change in a variety of fashions such as the molecule moving by translation or vibration, by nuclear spin changes, or by an electron being moved into a higher orbital. Visible and ultraviolet absorption spectroscopy involve electrons being knocked into different orbitals by the energy of absorbed photons. [1] Since the energy of a photon is dependent on wavelength, the differences between orbitals involve too large an energy change to produce infrared absorption. For infrared absorption, the energy change involves molecular vibrations. [2] Nuclear magnetic resonance involves changes in nuclear spin, which involve too small of an energy change to produce infrared absorption. [3]

2.2 Absorbance

Understanding absorption is important for this study because fluorescence can only occur after a more energetic photon has first been absorbed. The part of the molecule which absorbs the ultraviolet and visible wavelength photon is called a chromophore. Unlike infrared absorption which depends on vibrational energy level changes, visible
and ultraviolet absorption depends on changes between electronic energy states. The
different electronic energy levels of molecules are given by molecular orbitals which
result from combinations of atomic orbitals. An excited electronic energy level above the
ground energy level gives rise to a second Morse curve raised above the first. (Fig. 2.1)
For photon absorption to take place, an electron has to be moved from one of the vertical
vibrational levels on the first Morse curve representing one electronic energy level to one
of them on the second curve representing a higher electronic energy level. These curves
will also prove useful when discussing fluorescence. [1, 2]

The absorption bands are usually given with two parameters: the wavelength
position of the maximum and the extinction coefficient. The extinction coefficient is
determined by the Beer-Lambert Law, a simple exponential decay model dependent on
the depth into a substance. [1]

An example of absorption by highly conjugated systems is the porphyrin ring
system. It has a very strong absorption band at 420 nm called the Soret band and
additional absorption bands at 530 nm and 580 nm. A porphyrin ring is inside heme
molecules. [1]

2.3 Fluorescence

The parts of a molecule that can fluoresce are called fluorophores. Fluorescence
takes place after an electron is excited to a higher electronic energy level through
absorption of a photon, then the electron loses energy to reach the lowest vibrational level
within the excited electronic energy level, and finally the electron emits a photon as it
returns to the ground state electronic energy level. (Fig. 2.2) Since the electron spends a relatively long time in the excited state, about $10^{-6}$ to $10^{-9}$ seconds, it can lose its energy from the excited state in many competing ways besides emitting a photon. The primary competing loss mechanism is heat.

When fluorescence is induced in a sample by exciting at a single wavelength and the output is measured over a range of wavelengths, this is called an emission spectra. If instead the output is measured at a single wavelength while the excitation wavelength is altered, this is called an excitation spectra. [4] Doing both results in an Excitation-Emission Matrix. (EEM) Most often due to equipment and time considerations, emission spectra are taken with the excitation chosen for the fluorophore present. [4, 5]

### 2.3.1 Porphyrin as Both Dye and Natural Fluorophore

Research into using porphyrin dye to mark bladder tumors is relatively common, but using autofluorescence is more rare. The dyes used are hematoporphyrin and hematoporphyrin derivative (HpD), with the later the acetate derivative of the former. [6] The dye is most often administered as a drug and this can lead to side effects such as skin sensitization to sunlight for several days or even several weeks. [6, 7, 8, 9, 10, 11] When excited at 400 nm, it has emission peaks at 630 nm and 680 nm. [6, 12] A possible problem with the dye is it can become localized in inflamed or traumatized tissue and not just in tumors. [6] This dye was not used in the studies for this thesis.
Besides coming from dyes, porphyrins are created naturally in the body too and are contained within heme. [6, 12] Therefore, it is one of the potential natural fluorophores searched for in this study.

2.3.2 Natural Fluorophores in Tissue

Using autofluorescence, which doesn’t use dyes, removes the assumption that dyes will be absorbed in tumors and not in benign tissue. However, there are relatively few natural fluorophores present in tissue. Some that are present include aromatic amino acids (such as phenyladaine, tyrosine, and tryptophan), elastin, collagen, vitamin A, Y-base in t-RNA, nicotinamide adenine dinucleotide (NADH), flavins, porphyrins, and melanin. [4, 5, 7, 12, 13, 14, 15, 16, 17, 18, 19] Other than the Y-base in t-RNA, other nucleic acids don’t have appreciable fluorescence. [19]

The emission light wavelength best absorbed by tryptophan is 280 nm which produces a fluorescence peak at 348 nm. Tyrosine excites best at 274 nm with an emission peak at 303 nm and phenyaladaine at 257 nm with a peak at 282 nm. [19] The Y-base in t-RNA excites best at 320 nm with an emission peak at 460 nm. [19] Since the studies for this thesis excited at the considerably longer wavelengths of 370 nm and 400 nm, these amino acids and nucleic acid will not be important fluorophores.

Using a rat urinary bladder with chemically-induced dysplasia, Schomacker, et al., created an EEM with 6 excitation wavelengths and were able to distinguish between
neoplastic and non-neoplastic sites with an accuracy of 81%. The two most effective wavelengths were 370 and 415 nm. They suggest the discrimination might be from NADH and porphyrin fluorescence and from blood absorption. [5]

NADH excites best at around 360 nm and has its maximum emission at 450 nm. [1, 13, 15, 18] NADH is particularly interesting since when oxidized to NAD, it loses its fluorescence. Aubin found that NADH fluorescence matches well the autofluorescence from cultured mammalian cells when excited at 360 nm. [13] The 370 nm excitation used in this study is close enough to 360 nm that it should excite NADH if it is present.

Collagen will excite at 370 nm and produce an emission peak around 420 nm. [17, 20] Collagen when excited at 337 nm produces a peak at 390 nm, but shifting the excitation also shifts the emission peak. [21] Being more careful with terminology, it is crosslinking within the collagen that causes the fluorescence, but from here on collagen fluorescence refers to the fluorescence of the cross-linking of collagen. [22]

2.4 Light Scattering

Light scattering is due to induced oscillations caused in particles by the incident radiation. The oscillation reradiates the radiation in different directions. A simple model for this is an oscillating dipole. Because of this scattering, the light loses intensity, the intensity loss being called turbidity, and the light's apparent velocity is changed, called refraction. [23]
2.5 Light Spectra

Conventional spectroscopy uses a monochromator to directly produce the frequency spectrum. A monochromator uses a prism or a diffraction grating to produce a spectrum. This is the same principle used by Isaac Newton to turn white sunlight into the colors of a rainbow. Generally, a motor moves a mirror which then sweeps the different frequencies across the detector, so that the time axis of a plot of detector output will correspond to frequency. [2]

Since a computer is used to convert the interferogram into a spectrum, the detector must be connected to preamplifiers and an analog-to-digital (A/D) converter before being connected to the computer. Since the A/D converter will add quantization noise since it is only activated at discrete levels, it is desired to have the A/D converter reach close to its maximum value at the peaks in the interferogram to minimize the quantization errors which occur at low levels. [24]

2.6 Fiber Optics

Medical applications of optical fibers include performing laser surgery, acquiring data from sensors, and endoscopic visual inspections inside organs. [25, 26, 27, 28] Since many laser surgery applications involve acting on organs inside the body, both endoscopes and fiber optic sensors can help in the performance of laser surgery. [25, 28]
2.6.1 Medical Applications of Optical Fibers

2.6.1.1 Endoscopes

The main use of optical fibers in medicine is through a device called an endoscope. An endoscope is a tubular instrument used in examining body cavities or organs. The earliest endoscopes were simply narrow rigid pipes without any fiber optics at all. One of these pipes was inserted into an organ such as the esophagus or rectum. The physician would stare down the tube. [28] Most modern endoscopes are flexible devices which utilize either an ordered bundle of fiber optics for producing an image or a minuscule videocamera, but even the rigid endoscopes includes lens, cameras, or optical fibers rather than simply being a tube. [25, 28, 29] Endoscopes often have other names based on their specialized application such as the bronchoscope, for examining the trachea and large bronchi of the lungs; the cystoscope, for examining the urinary tract; and the otoscope for examining the outer ear canal and the eardrum. [25] Thus, this *in vivo* bladder study used a cystoscope.

A typical modern endoscope incorporates two optical fiber bundles. (Fig. 2.3) The illuminating bundle carries light from a high intensity source such as a Xenon arc lamp to tissues within the body. The imaging bundle transmits images from the illuminated tissue back to the observer. [25] While the relative fiber placement in the illuminating bundle is unimportant, the imaging bundle requires that the individual optical fibers have the same spatial placement at both ends so the input image into the bundle is reconstructed properly at the output. [25] Since an imaging bundle can contain 10,000 fibers within a
diameter less than one millimeter, the image will have high spatial resolution. In some modern endoscopes, miniature CCD cameras mounted at the endoscopes tips replace the imaging bundle.

Endoscopes often have one or more auxiliary channels. The auxiliary channel is merely a hollow cylinder which extends down the length of the endoscope. This hollow tube allows the taking of biopsies by inserting a wire with a tiny clamp attached to the end to snip off a tissue sample. In a similar fashion, the auxiliary channel allows cutting of tissue by inserting a tiny scalpel at the end of a wire. The hollow tube can also be used to inject or remove fluid. For this study, the optical fiber probe is inserted down the endoscope's auxiliary channel and the tip of the optical fiber probe is brought with light pressure against the wall of the organ. The CCD camera in the tip of the flexible endoscope or the telescopic lens arrangement in the rigid cystoscope (a nonflexible type of endoscope used in the bladder) allowed viewing of the extended optical fiber probe to see that good contact was made with the tissue surface.

2.6.1.2 Sensors

A variety of sensors have been put on optical fibers to probe and measure parameters of the human body. Here is a list of a few measurements which can be taken with sensors attached to optical fibers: blood pressure can be determined by looking at reflections from a flexible diaphragm; blood flow can be calculated using the Doppler effect; pulmonary artery oxygen content can be estimated based on reflected spectra; and pH can be checked by examining organic dyes encased in a porous polymer affixed to the
For this study, there is not a sensor attached to the distal end of the optical fiber probe inserted into the body. Instead, excitation light to create fluorescence travels down one optical fiber and then the returned emission light from tissue fluorescence travels back down six other optical fibers to a spectrometer. So, the sensor is in the instrument outside the body while the optical fiber probe transports the light into and back out of the body to where the sensor can measure it. An alternative perspective is to include the optical fiber probe as an integral part of the sensor for measuring spectroscopy from locations inside the body.

For laser surgery applications, it may be beneficial to distinguish between healthy tissue and cancer or between normal artery tissue and atherosclerotic plaque. For finding atherosclerotic plaque instead of normal artery tissue, fluorescing spectra are being taken and analyzed at various excitation wavelengths. This is necessary for laser angiosurgery which attempts to cut away, i.e. ablate, atherosclerotic plaque without causing damage to the artery such as perforation.

For finding cancer in the bladder, one technique not used in this study but related to it is for a hematoporphyrin derivative dye to be injected into a patient days earlier. This can be used to locate tumors because tumors absorb the dye much more quickly than normal tissue. Under UV illumination, the dye fluoresces with red light. This is being tested clinically. With this study, no dyes are used, but porphyrin molecules also occur naturally in the human body so may be part fluorescing structures (fluorophores) creating the measured autofluorescence spectra of bladder tissue. [19]
2.7 Lasers

Various properties affect the type of interaction that takes place between laser light and tissue. These properties involve factors such as the type of laser, the spot size, and the type of tissue. [28]

2.7.1. Wavelengths Absorbed by Tissue

The absorption spectra for many types of tissue vary considerably with wavelength. Biological tissue usually has absorption spectra with various peaks. Chromophores, which are substances that determine the color of a compound, alter the spectra and produce these peaks. Chromophores in the human body include hemoglobin, a major component of blood; melanin, which determines skin darkness; and keratin, a major component of hair. [25] Even simple water acts as a chromophore at infrared wavelengths. [32] If a laser is operated at or near these absorption peaks, the penetration depth will be small and the absorbed energy within that depth will be great. This is desired for cutting or concentrating heat in a small area. [32] For example, the visible light of the Argon laser is mainly absorbed in pigmented tissues including blood. This laser radiation works well to coagulate blood to stop bleeding. [28] On the other hand, the 10.6 μm infrared radiation of a carbon dioxide laser is mainly absorbed in water and works well for cutting water-containing tissues as well as bone. [28, 33, 34] (Fig. 2.4)

For the visible wavelength region from 400 through 700 nm, an important absorber in biological tissue is hemoglobin, whether oxidized or deoxidized. [1, 17, 20,
The large 415 nm absorption peak for oxidized hemoglobin and the large 420 nm peak for deoxidized hemoglobin is called the Soret band. [1, 35, 36] In addition, for oxidized hemoglobin there are also peaks at 530 and 580 nm while for deoxidized hemoglobin there is a broad peak at 555 nm. [35, 36]

### 2.7.2 Wavelengths Dangerous to Living Tissue

For medical applications, one concern is that wavelengths from 280 to 320 nm are mutagenic; i.e., they are effective in inducing tumors. This has been demonstrated in animal studies using both conventional UV lamps and lasers. [31] This is likely to be due to damage to the bonds in DNA molecules from photochemical interactions with the UV radiation. [31]

In this studies for this thesis, 370 nm excitation light, 400 nm excitation, and white light (400 nm through 700 nm) were used, avoiding the mutagenic region.

### 2.7.3 Laser Selection

For laser surgery applications using an optical fiber, a laser must be chosen which produces a wavelength which takes into account selective absorption in the proper tissue; avoidance of mutagenic potential; and acceptable power density transmission through the optical fiber. Different types of lasers produce various wavelengths of light. For example, some common lasers for laser surgery are carbon dioxide which produces light at 10.6 μm, Nd:YAG for 1.064 μm, Argon for lines at 488 and 514 nm, tunable dye lasers which cover the visible spectrum, and excimer lasers which are in the ultraviolet. [28]
This list covers some lasers commonly used, but many other types of lasers available and used too. [31, 37] For this study, a tunable nitrogen dye laser was used and set at 370 nm excitation and 400 nm excitation. Both wavelengths were derived using a single laser dye (Exiton BBQ), producing an average power of 250-350 $\mu W$ delivered to the probe tip in 3 ns pulses at 10 Hz.

**2.7.4 Beam Dimension**

The beam dimension affects the tissue response. A simple example is that of a lens used to concentrate sunlight to cause a sheet of paper to ignite. The lens position must be adjusted so that the spot size is minimized. It is not the delivered energy itself which raises the temperature of the paper enough to ignite, but the energy divided by the area of the spot. This is called the energy density or fluence. The same is true with laser interactions with tissue. [31] For the simple example of igniting paper with sunlight and a lens, the energy must be delivered to the paper quickly enough so that the temperature of the paper can't equalize. The rate of energy delivery is power. Therefore, power density (power per area) can also be useful. Both fluence and power densities are frequently discussed. [31, 38] A useful figure has power density (W/cm$^2$) on the vertical axis and interaction time (seconds) on the horizontal axis. [33] (Fig. 2.6) A line with a negative slope on this graph gives the fluence with the border for having any chance of doing physical damage at approximately 1 mJ/cm$^2$, whether the damage is from electromechanical, a photoablative, a thermal, or a photochemical response. With 300 $\mu W$ producing a 750 $\mu m$ spot size has a power density of $6.8 \times 10^4$ W/cm$^2$ with an
interaction time of $3 \times 10^{-9}$ seconds. This is to the far left on the graph, far below the values which produce physical damage. It has a fluence of $2.0 \times 10^{-7}$ mJ/cm$^2$, much less than 1 mJ/cm$^2$.

2.8 Phase Contrast

Light scattering is determined by a substance’s turbidity and refraction. Some materials can have similar turbidities but different refractions. For these types of materials, such as frozen and then thawed thin tissue sections, there will be little observed difference on a regular microscope because the light absorptions, as shown by the similar turbidities, will be about the same. However, if only light refracted through the materials is observed, then the materials will look quite different. The phase-contrast microscope does this. [23]

The phase-plate annulus, although it looks black under a phase-contrast microscope’s eyepiece, is not completely opaque. It is a phase-plate which changes the phase of the light transmitted through it by 90 degrees. Scattered light also has a 90 degree phase shift compared to the incident light. This means that there is a 180 degree difference between the scattered light, which doesn't pass through the phase-plate annulus, and the directly transmitted light, which does pass through the phase-plate annulus. This provides cancellation. However, the transmitted light would be overly bright compared to the scattered light, so the phase-plate annulus includes a thin metal film along with the phase-plate. The metal film reduces the intensity of the directly transmitted light so that it matches that of the scattered light. [23]
REFERENCES


horizontal lines represent allowed vibrational levels

Morse curve for first excited electronic state

Morse curve for ground electronic state

Figure 2.1: Two electron energy levels as Morse curves demonstrating light absorption.
horizontal lines represent allowed vibrational levels

Morse curve for first excited electronic state

Morse curve for ground electronic state

Figure 2.2: Two electron energy levels as Morse curves demonstrating light fluorescence.
Figure 2.3: Schematic diagram of a typical endoscope.
Figure 2.4: The wavelength-dependent absorption of water and bone. [33]
Figure 2.5: Oxidized and deoxygenized hemoglobin absorption. [35]
Figure 2.6: Laser-tissue interaction categories. [38]
CHAPTER 3

EQUIPMENT

3.1 Spectroscopy

A system for spectral measurements was built on a mobile cart which can be wheeled into the operating room. The excitation light was from a nitrogen-pumped dye laser (Laser Science VSL-337ND nitrogen laser and DLM-220 dye laser module). [1, 2] (Fig. 1) A single laser dye (Exiton BBQ) was chosen which was tunable to both 370 nm (near UV) and 400 nm (deep blue).

A small optical fiber bundle fashioned into a probe was placed down the auxiliary channel of an endoscope or cystoscope. (Fig. 1 and 2) Having the optical fiber bundle fit in the auxiliary channel is common in many studies. [1, 2, 3] The probe had a single 200 \( \mu \text{m} \)-core, 0.22 NA excitation fiber surrounded by an array of six 200 \( \mu \text{m} \)-core, 0.22 NA collection fibers. (Fig. 1.) At both 370 nm and 400 nm excitation, an average power of 250-350 \( \mu \text{W} \) was delivered to the probe tip in 3-ns pulses at 10 Hz. A 750 \( \mu \text{m} \)-diameter excitation spot was produced at the tissue surface.

Fluorescence emitted by the tissue was transported up the collection fibers and focused at the entrance slit of a spectrograph (Instruments SA CP-200) coupled to an MCP-intensified optical multi-channel analyzer (EG&G PAR OMA III). [1, 2] A 399 nm
or 418 nm long pass, low fluorescence filter blocked scattered 370 nm or 400 nm excitation light from the detector. A 1.0 μs collection gate synchronized to the laser pulse allowed detection of weak tissue fluorescence during illumination. The spectral resolution was 6.3 nm. [2] A total of 40 spectra, ranging from 400 nm (deep blue) to 700 nm (far red), were collected at each tissue site (4 sec total acquisition time) and summed to maximize the signal-to-noise ratio. This resulted in a signal-to-noise ratio of greater than 60:1 at wavelengths shorter than 600 nm. Rejection of stray excitation and emission light by the spectrograph, especially in the red region of the spectrum, was confirmed by examining the emission spectrum of a small amount of Coumarin 440 in methanol (approximately 10⁻⁴ M) on a highly scattering BaSO₄ plate. Less than 0.3% of the peak fluorescence intensity at 443 nm could be observed at 600 nm, and less than 0.05% at 700 nm.

For each in vivo patient, spectra were taken at two to four normal and two to four papillary tumor sites. For each in vitro bladder removed from the patient less than thirty minutes, six to eight sites were measured. (Fig. 3) The optical fiber probe was held in contact with the tissue surface such that the tip displaced bladder contents or blood. Excitation from the dye laser was performed at one wavelength for all sites, then the laser was tuned to a different wavelength and spectra were taken again.

At each site, biopsies were taken and immediately placed in Hollande’s fixative. These were processed into hematoxylin-and-eosin (H&E) stained sections and evaluated by a single pathologist at The Cleveland Clinic Foundation.
Some small modifications were made to the system for the in vitro study. A flashlamp (EG&G flashtube model FX-249) was added for providing white light so reflectance spectra could also be taken. A beamsplitter constructed using the reflection from a glass cover slip inserted the light from the flashlamp into the optical path of the laser and optical fiber probe. The probe’s distal end had a 15° angle cut at its tip to greatly reduce reflection from its own end. The flashlamp, like the laser, was be triggered at 10 Hz, so the detector software would operate properly without any changes.

In order to compare spectra acquired from different patients at different times, each spectrum was calibrated in a standardized manner. First, a measured background spectrum was subtracted to correct the spectral baseline to zero. Variations in laser power, collection efficiency, and system alignment were then eliminated by dividing each spectrum by the fluorescence intensity of a 3 mm-thick 485 nm colored glass long-pass filter measured at each excitation wavelength. Finally, the nonuniform spectral response of the detection system was corrected using the measured spectrum of a calibrated tungsten-halogen lamp.

The spectra were acquired on a personal computer (Everex 386/33) using OMA 2000 software (Princeton Instruments).

Much of this equipment is the same or derived from that used by Cothren et al. in colon studies. Differences include running at 400 nm excitation as well as 370 nm, installing a flashlamp and related optics for reflectance measurements, using a different optical fiber probe, altering the timing for 10 Hz rather than 20 Hz operation, and measuring a different reference for calibration. [1, 2]
3.2 Microscopy

For the excitation light, a 75-W Xenon arc lamp fitted with a 380 nm or 400 nm narrow-band interference filter was coupled to microscope slides on the stage of an Olympus IMT-2 inverted microscope. (Fig. 4) An IR filter (Oriel Model 61945) between the arc lamp and the interference filter prevented thermal damage. (Fig. 5) The emitted fluorescence traveled on to form an image on a Princeton Instruments thermoelectrically-cooled CCD camera (Model TE/CCD-500B with an ST-130 controller). (Fig. 4) Reflected excitation light was prevented from reaching the camera by a long-pass barrier filter. The camera was cooled to -45°C to reduce leakage current and thus background noise. The 518x384 pixel images have a resolution of 1.2 μm and a dynamic resolution of 16-bits [4]. A gray scale fluorescent image was taken. Since the CCD camera is not a color device, when color images were desired, three separate images were collected using an additive red-green-blue filter set (Andover Corporation, Set 126FA44-25). These were combined into a single color image.

To provide nearly Kohler illumination at the microscope stage, the parabolic reflector behind the arc lamp acted as the lamp lens and an added quartz glass lens (f50) served as a condenser. An adjustable one-inch aperture was also added to act as the iris diaphragm. [5, 6, 7] Kohler illumination collimates the light illuminating the object.

Frozen biopsy specimens were cut into 5 μm sections. Unstained frozen slides were imaged on a fluorescence microscope while serial sections were stained with hematoxylin-and-eosin (H&E) for review by a pathologist.
Tissue orientation was vital for later creation of the fluorescent density function. The tissue was oriented when cut so that the slices were perpendicular to the mucosal surface. Then, on the microscope, the slide was oriented so the horizontal axis of the image matched depth into the tissue and the vertical axis was parallel to the mucosal surface. This orientation allowed integration along the vertical axis to get a fluorescence density function based on depth in the tissue. [4]

When producing a black-and-white, background-subtracted, flat-fielded fluorescence microscope image, an image is taken of the sectioned tissue on a microscope slide, another of a blank slide for the background including PBS solution and a cover slip, and yet another of an isotropic fluorescent object for a reference (STC Fluorescent Standard FL-160, machined to a thickness of 200 $\mu$m). Subtracting the background greatly reduces the effects of leakage current and lens fluorescence and then dividing by the reference image corrects for nonuniformities in both the illumination and the CCD camera to provide a flat viewing field.

For color images, an Andover dichroic additive color filter set passes the red, green, and blue spectral components resulting in three sectioned tissue images. Background images are collected with these same three filters and then subtracted from the other images. The same reference image is divided into these images, resulting in three background-subtracted, flat-fielded images. For finding color balance, three images are also collected of reflections from the relatively spectrally-flat arc-lamp measured.
without an excitation bandpass filter and the average intensities are calculated so the red, green, and blue tissue images can be combined into a single background-subtracted, flat-fielded, color-balanced, fluorescence color image.

The electronic images acquired on a IBM PC clone (Everex Tempo 486/33c) with WinSpec software (WinSpec Version 1.3, 1995, Princeton Instruments). (Fig. 6) The images were then transferred to a Sun computer (Sparstation IPX) for further processing. The Khoros visual programming system (Version 1.05, Copyright 1991, University of New Mexico) was used for the background subtraction, flat-fielding, and combining of red, green, and blue images into one color image.
REFERENCES


Figure 3.1: Schematic diagram of the laser spectrofluorometer, illustrating the distal tip of the optical-fiber spectral probe in the inset.
Figure 3.2: Optical fiber probe with the tip against a colon polyp after being inserted down the auxiliary channel of an endoscope.
Figure 3.3: Removed bladder prepared for spectral measurements.
Figure 3.4: Schematic diagram of the fluorescence microscopy equipment.
Figure 3.5: Xenon arc lamp, IR filter, bandpass filter, and diaphragm for illuminating tissue on microscope stage.
Figure 3.6: CCD camera and personal computer connected to the Olympus microscope.
CHAPTER 4

MATHEMATICAl ANALYSIS

4.1 Comparison to a Gold Standard

To determine if a new test produces valuable results or not, it has to be compared to a gold standard. This could come from examining a biopsy, autopsy results, or comparing to an established test. The gold standard is needed to determine diagnostic utility, but it does not address whether the new test could be better than the gold standard. [1, 2]

For the in vivo colon tissue and in vivo and in vitro bladder tissue studies discussed in this thesis, the gold standard was from biopsies taken from the tissue, sectioned, stained with hematoxylin and eosin (H&E), and then reviewed by a pathologist.

An issue that arose when comparing bladder in vivo and in vitro studies was the importance of the reference population. Even with a test that compares well to the gold standard on one particular reference population may not work well on a different population. Consider a test for the range of normal hematocrit created by testing only men. It may not apply well to women because they tend to have a lower hematocrit overall. [2] Another example is a test for a disease may be created using medical students
as subjects due to their ready availability. However, the medical students tend to be fairly young so the results from testing them may not apply when the test is performed on those of retirement age. [2] It turned out in the studies for this thesis, the *in vivo* and the *in vitro* bladder studies have very different patient populations due to the patient selection process. The *in vitro* patients were having their bladders removed because of bladder cancer, indicating bladders at an advanced stage of disease. In contrast, the *in vivo* patients were being examined for papillary tumors, a superficial tumor type, which if found were removed cystoscopically although they would have had little risk of progressing to invasive cancer even if not removed.

To gage the results of the new test, sensitivity and specificity were calculated. Sensitivity is the proportion of those with disease according to the gold standard also claimed by the new test to have disease. Specificity is the proportion of those with non-disease according to the gold standard also claimed by the new test to have non-disease. [2] Also used are the predictive value of a positive test and the predictive value of a negative test. The former is the proportion of those which the new test claims have disease who according to the gold standard do have disease. The predictive value of a negative test is the proportion of those with non-disease according to the new test who actually according to the gold-standard do have non-disease. [2] To easily calculate the sensitivity, specificity, and predictive values, a table can be constructed which includes True Positives (TP), False Positives (FP), False Negatives (FN), and True Negatives.
(TN). (See Table 4.1 and Table 4.2 for notation) [1, 2] For percentages for sensitivity, specificity, predictive value of a positive result (PVP), and predictive value of a negative result (PVN), simply multiply by 100.

<table>
<thead>
<tr>
<th>New Test Being Considered</th>
<th>Gold Standard (Pathologist)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease</td>
</tr>
<tr>
<td>Test says disease</td>
<td>TP</td>
</tr>
<tr>
<td>Test says non-disease</td>
<td>FN</td>
</tr>
<tr>
<td>Summation</td>
<td># with disease</td>
</tr>
</tbody>
</table>

Table 4.1: Results of gold standard and new test.

The following equations calculate sensitivity, specificity, PVP, and PVN: [1, 2]

\[
\text{sensitivity} = \frac{TP}{TP + FN} \quad (1) \\
\text{specificity} = \frac{TN}{TN + FP} \quad (2) \\
PVP = \frac{TP}{TP + FP} \quad (3) \\
PVN = \frac{TN}{TN + FN} \quad (4)
\]

While the sensitivity and specificity predict the percentages correctly categorized for the reference population, this may not be the actual results if tested under for a different population set. To determine that, the frequency of the disease in the new group or prevalence for that new group is required. [1, 2] At 400 nm excitation, nine out of the ten patients had papillary tumors for the in vivo study, but only two out of thirty patients had papillary tumors for the in vitro study. For the in vitro case with the few patients
with papillary tumors, the sensitivity was low but the specificity was extremely high. By adjusting the decision threshold, sensitivity and specificity can be traded off one for the other. Two methods were used to adjust the decision threshold. The first method was to weight the decision threshold by the percentage of those having the "non-disease" and "disease" so it would act like each category appeared roughly the same percentage. The second method was to use receiver operating characteristic (ROC) curves and pick the point on the curve closest to the optimum. ROC curves discussed in the section below.

4.2 ROC Curves

Receiver operating characteristic (ROC) curves show the result of getting greater sensitivity at the expense of specificity and vice versa as the decision threshold is adjusted up and down. (Fig. 4.1) [1, 3] The vertical scale for the ROC curve is the true-positive rate which is identical to the sensitivity. The horizontal scale is the false-positive rate, which is 1.0 minus the specificity. An inserted 45° line represents what the result would be for perfectly random data. For example, 50% sensitivity and 50% sensitivity would be perfectly random. However, by trading off sensitivity and specificity, a 90% sensitivity with a 10% specificity would also be perfectly random. [1, 2] The 10% specificity would give a 90% false-positive rate so that along with a 90% sensitivity would be on the 45° line.

Thus, giving solely the sensitivity or specificity does not give a complete picture of the results of a test. Perfect results would be 100% sensitivity and 100% specificity, but 100% of one without specifying the other tells little about the performance of the test.
A result might be assumed at 90% or 95% if a figure is not given, but this might not be a correct assumption. The 100% sensitivity and 100% specificity point is at the upper left corner of the graphing space for the ROC curve or the (0,1) point. Most ROC curves are between the two extremes of perfectly random on the 45° line and the (0,1) optimum point. The further the curve gets from the 45° line and the closer to the upper left corner, the better the results. [1] On a graph, the distance from the ROC curve from the 45° line and the closest point to the (0,1) optimum point is easily visualized. The point on the ROC curve closest to the (0,1) optimum point can be calculated by calculating the Euclidean distance from it to all the points on the curve, d, and choosing the smallest.

\[ d = (1 - \text{sensitivity})^2 + (1 - \text{specificity})^2)^{0.5} \]  

(5)

4.3 Bayes’ Theorem

When comparing the colon fluorescence results between the principal component analysis with logistic regression method (PCA/LR) to an earlier method using the normalized 460 nm peak with the normalized ratio of 680 to 600 nm developed by Cothren et al. as a test to evaluate the performance of the PCA/LR method, Bayes’ Theorem came into play. [4, 5] Cothren’s colon article expresses Bayes’ Theorem as: [4]

\[ p_j(x, y) = \frac{p_j f_j(x, y)}{\sum_j p_j f_j(x, y)} \]  

(6)
Variables and Parameters:

\((x, y)\): measured values.

\(x\): normalized 680 nm/600 nm.

\(y\): normalized 460 nm peak.

\(j\): various categories numbered one on up. In this case, 1 for normal colon tissue, 2 for hyperplastic polyps, and 3 for adenomatous polyps.

\(P_j(x,y)\): probability of measured values indicating type \(j\).

\(p_j\): probability of finding category \(j\) in sample population.

\(F_j(x,y)\ dx\ dy\): probability something of category \(j\) will have values \((x,y)\).

Bayes' Theorem can be written in a simpler form when there are only two categories such as non-diseased and diseased. It can be written out in text as the conditional probability, which is the desired answer, being equal to: [1]

\[
P = \frac{\text{sensitivity} \cdot \text{prior probability}}{\text{sensitivity} \cdot \text{prior probability} + (1 - \text{specificity}) \cdot (1 - \text{prior probability})}
\]

Written this way, terms in the numerator and denominator require the assumption of a prior probability which affects the final result. Another phrase used for the prior probability is the index of suspicion. The prevalence of the disease may be used as the prior probability. [1] For example, if it is assumed beforehand that roughly half the patients in a study have a particular disease and half do not, that gives a very different prior probability then if it is assumed beforehand that only one patient out of a hundred
will have the disease. Bayes' Theorem uses this prior knowledge to get a more meaningful answer more quickly and also to relate the results of one reference population when applied to a different population with a different set of prior probabilities. [1]

However, with only two categories, the previous section on ROC curves demonstrates how sensitivity and specificity can be traded off. Bayes' Theorem can use the observed prevalence as the prior probability gives one point on the ROC curve. Results not weighted based on any prior probability give another point on the ROC curve. Results with the decision threshold weighted to force the size of the non-diseased and diseased sets to act as if equal in size give yet another point on the ROC curve. Results from following the ROC curve while calculating the shortest Euclidean distance to the (0,1) optimum point gives still yet another point. For the purposes of seeing how the tests functioned in this thesis, the point on the ROC curve the shortest distance to the (0,1) optimum point was the preferred method.

When there are more than two categories, trading off thresholds to gain in one category at the expense of another becomes problematic. The ROC curve may not be exist because with more than two categories, the meaning of the terms sensitivity and specificity may not be obvious since they could be referring to different categories. For example, if one category is dysplastic tissue, it might be considered in the diseased category if one considers abnormal tissue as diseased but it might be in the non-diseased category if one considers only malignant tissue, which dysplastic tissue is not, as diseased. Then again, dysplastic category might be put in its very own category separate from non-diseased tissue and other types of diseases. For multiple categories, using
Bayes' Theorem with chosen prior probabilities can provide more meaningful answers than not including the prior probabilities. For this thesis, results were calculated with ROC curves with only the two categories of non-diseased and diseased with the diseased category altered for many tests to try various combinations. For example, in the in vitro bladder reflectance study, one test was performed with dysplastic tissue in the non-diseased category and another test with it in the diseased, then the results were compared which demonstrated considering dysplastic tissue as diseased resulted in better performance.

4.4 Calibration and Validation Sets

Using the entire set of experimental data in one large set and analyzing it with a model produces explained variation. However, for making diagnoses, what is required is not explained variation, but the predictive value of the model. For calculating that the model must be tested on data which was not used to create the model itself. That is, to avoid bias, the diagnostic algorithm should not be developed and tested on the same data set used to create it but instead use a calibration or training set to create the model and a validation or testing set for evaluating it. [6, 7, 8, 9, 10, 11, 12, 13, 14]

4.5 T-tests and P-values

To determine if two sets of quantities are different to a degree that is statistically significant, a common technique is calculating the p-values from a two-tailed student's t-test assuming equal variance. In statistical terms, the $H_0$ hypothesis being tested is that
the two sets are equal to each other with the alternate $H_1$ hypothesis being that they are not equal. This is a standard test described in most statistics books and a common assumption made is having a p-value at or under 0.05 indicates the values are significantly different. [2, 15, 16] For the t-test, the first step is to calculate the pooled variance, $s_p$. [16]

$$s_p = \sqrt{\frac{\left(\sum_{i \in A} (x_i - \langle x_A \rangle)^2 + \sum_{i \in B} (x_i - \langle x_B \rangle)^2\right)}{N_A + N_B - 2} \left(\frac{1}{N_A} + \frac{1}{N_B}\right)}$$ \hspace{1cm} (8)

Variables and Parameters

$s_p$: pooled variance.

A: Set of quantities for non-diseased sites.

B: Set of quantities of diseased sites.

$N_A$: Number of non-diseased sites.

$N_B$: Number of diseased sites.

$\langle x_A \rangle$: mean of non-diseased sites.

$\langle x_B \rangle$: mean of diseased sites. [16]

Once the pooled variance is calculated, the t-test itself can be performed: [16]

$$t = \frac{\langle x_A \rangle - \langle x_B \rangle}{s_p}$$ \hspace{1cm} (9)
With the t-test performed, the p-value can then be calculated, requiring the additional information of the degrees of freedom, v: [17]

\[
p(t|v) = \frac{1}{v^2 \beta \left( \frac{1}{2}, \frac{v}{2} \right)} \int_{-\infty}^{t} \left( \frac{x^2}{v} \right)^{-\frac{v-1}{2}} dx \quad (10)
\]

\[
with \quad \beta \left( \frac{1}{2}, \frac{v}{2} \right) = \int_{0}^{1} y^{\frac{v}{2}-1} (1-y)^{-\frac{1}{2}} dy \quad (11)
\]

Many spreadsheets and statistics packages include t-test functions which report p-values. For this thesis, the t-test routine from Microsoft Excel, TTEST, was used with options selected for a two-tailed test assuming equal variance.

Having a parameter pass a Student t-test at the 0.05 significance level indicates a significant difference in the chosen parameter, but indicates little about its usefulness for diagnostic purposes. Numerical Recipes in C gives the amusing example, "A treatment for baldness has caused some patients to lose all their hair and turned others into werewolves, but we want to know if it helps cure baldness ON THE AVERAGE!" [16] Another amusing example is the average height of male adults is different than the average height of female adults and with a sufficiently large sample size will pass a t-test at the 0.05 significance level, but to use height as a diagnostic test for being male or female will nevertheless work poorly due to the large variance in people's heights. A difference can be statistically significant without being too useful as a diagnostic test.
A simple numerical example of this concept can be given. Create one ten-element array with the values 1, 2, ..., 10. These ten values will be given the label of disease. Create a second ten element array with the values 4, 5, ...13. These will be called non-disease. This can be plotted. (Fig. 4.2) The wide variance with extensive overlap looks similar to the fluorescence intensity graph of the 460 nm emission values with 400 nm excitation in the bladder *In Vitro* fluorescence spectroscopy chapter.

Using a decision threshold of 6.9 results in 60% sensitivity and 70% specificity. Alternatively, a decision threshold of 7.1 results in 70% sensitivity and 60% specificity.

For the first array, the mean is 5.5 and the standard deviation is 3.0. For second array, the mean is 8.5 and the standard deviation is also 3.0. When two Gaussians are plotted with these means and standard deviations, the overlap of area is between 30 and 40%! (Fig. 4.3) This is easily understood since one standard deviation away from the mean for a Gaussian is 68.3% and the two means are about one standard deviation away from each other.

The two-tailed t-test assuming equal variance for these two arrays results in a p=0.04 which is lower than 0.05 so passes the commonly used significance test. [16] Passing the p-value at the 0.05 level does not mean the distribution of the samples themselves overlaps only that much, but rather the 0.05 refers instead to the test of the hypothesis that the two sets have equal means and that is the probability distribution under question.

The point of this exercise is that while 60% sensitivity and 70% specificity is better than random chance, they are not likely high enough for medical diagnostic
purposes. Therefore, passing a t-test at the p<0.05 significance level does indicate a significant difference, but does not show if that difference can be exploited for a meaningful diagnostic test. This distinction is important to realize because many articles on bladder tissue fluorescence and reflectance spectroscopy discussed in the other chapters give p-values to show diagnostic potential, but that is extrapolating beyond what the p-values computed from t-tests actually determine. [1]

4.6 Cross-validation

For a large data set, the set can be split in half with one half designated the calibration set and the other half the validation set. A diagnostic method can then be run with calculations on the calibration set to create a diagnostic model and the capabilities of that resultant diagnostic model can be used on the validation set with the result for the validation set providing the sensitivity and specificity. To be careful and complete, the calibration and validation set can then be reversed and the process repeated. With a sufficiently large and adequately random total sample set, reversing the sets should cause little change in sensitivity and specificity. If there is a large change in sensitivity and specificity when the sets are reversed, as was found when this procedure was done on the bladder *in vivo* fluorescence data set at 400 nm excitation which had only ten patients, then simply splitting the total set in half may be inadequate with the cause likely to being a total set which is too small. Taking more experimental data should eventually cure that problem.
An alternate method of creating a calibration and validation data set when the overall data set is small is instead to use a cross-validation scheme which optimizes the use of the data while still satisfying the need for each calibration set to be independent from the validation set. [3, 7, 8, 18, 19] This involves the creation of many calibration and validation sets with a diagnostic model created for each pair of sets. Cross-validation is equivalent to the leave-one-out (LOO) method, where each algorithm is created with one set and tested on another set and then cycled through the entire set. The final result provides the predictive value of the model. [8, 14, 15]

In our cross-validation scheme, the entire data set with one patient excluded was used as the calibration data set to develop a diagnostic algorithm, the results of which were then applied to diagnose the sites of the excluded patient. The process was continued with the excluded patient's data put back and another patient's data then excluded until the data from all the patients was cycled through. This meant each created diagnostic algorithm was tested on data to which it had been blinded.

Since the intensity calibration signal and background signal were taken per patient rather than per emission spectrum, any errors in them affect not just one measured emission spectra, but all for that patient. Therefore, we excluded the data from one patient rather than just one spectrum to prevent intra-patient bias, insuring that the diagnostic algorithm is robust to a multi-patient sample. If it is assumed that each patient has a similar number of sites, our method is comparable to k-fold cross-validation. [19]
4.7 Spectral Analysis

4.7.1 Normalizing to Patient's Own Non-Diseased Tissue

The intensity calibration of spectra can be problematic. The experimental technique for this the studies in this thesis was for an optical fiber probe to pressed lightly against the tissue. The probe may not have been perfectly perpendicular to the tissue or to the reference surface for calibration. Contact between the probe and the surface may be poor. A technique to deal with intensity calibration problems as well as to compensate from some of the natural intra-patient variability is to normalize all the spectra. One common method for normalization is to set the area under the curve to exactly 1.0. Another common method is to set the maximum peak height to exactly 1.0. Both of these have the disadvantage of removing or reducing information contained in the overall change in intensity. This could be removing important diagnostic information because a commonly reported result for tissue fluorescence, as discussed in the first chapter of this thesis, is a decrease in amplitude as the tissue becomes more diseased. To keep the information contained in this intensity change while still normalizing, a different normalization procedure is available, that of normalizing to the patient's own non-diseased tissue. [4, 20, 21, 22, 23] With this technique, it is assumed one has prior knowledge of what tissue is non-diseased and what tissue is diseased. For each patient, each extracted emission value or each entire emission spectrum is divided by the mean extracted emission value or the entire spectrum of the non-diseased tissue for that patient.
For example, let us assume each patient has exactly two sites, one site being diseased and one site non-diseased. Let us further assume that each has an emission peak at 460 nm and that the peak for diseased tissue is generally lower than that for non-diseased tissue. Papillary tumors protrude from surface of the bladder wall and can almost always be seen, so that if one has only flat non-diseased tissue and papillary tumors in a study, that is a situation where the diseased tissue can be determined by gross observation with reasonable accuracy even before the pathologist has reviewed the biopsies. Since for this hypothetical example, there is only one non-diseased tissue site for each patient, that spectrum is the mean spectrum for the non-diseased tissue for that patient. Taking the ratio of the identical spectra will be a flat line with a value of exactly 1.0. If the single diseased tissue spectrum for that patient has exactly the same shape but a lower overall amplitude, dividing it by the non-diseased tissue spectrum will produce a flat line with a value less than 1.0. If the single diseased tissue spectrum for that patient has different spectral features, the line after dividing will not be flat. Taking the value of the spectra at 460 nm, the result for non-diseased tissue in this hypothetical example will always be exactly 1.0. Since it is assumed for the hypothetical example that the peak amplitude for diseased tissue generally is lower, that will generally have a value less than 1.0.

Now, assume the spectra for the hypothetical example above were consistently and meaningfully intensity calibrated and assume the optical properties of the non-diseased tissue was about the same between different patients and also that the diseased tissue spectra were generally lower in amplitude. Then, simply taking the emission peak
and choosing a threshold between non-diseased and diseased would tend to work well even without normalizing to the patient's own non-diseased tissue. However, now assume the spectral intensity from non-diseased tissue varies greatly from patient to patient because the non-diseased tissue itself varied greatly from patient to patient. Even if the non-diseased tissue spectra is consistently higher in amplitude then the diseased tissue spectra for each specific patient, then the results of a simple universal threshold applied to all the patients will likely be poor. Similarly, if the intensity calibration is from poor patient to patient, that can also make the results of a simple universal threshold applied to all the patients be poor also. Normalizing to the patient's own non-diseased tissue essentially used the patient's own non-diseased tissue to perform the intensity calibration and thereby adjusts for calibration constant errors and compensates for some of the differences in the non-diseased tissue between patients.

However, normalizing to the patient's own non-diseased tissue only has meaning if a priori knowledge is used. If a pathologist reviews the tissue and then his or her diagnoses are used to determine the non-diseased tissue for normalizing, then that normalization already incorporates information supplied by the pathologist and biases the data. Consider the same hypothetical example of two sites per patient with always one non-diseased site and one diseased. Imagine before normalization that the resultant spectra for both non-diseased sites and diseased sites are completely random with the same mean and variance. Since each patient has only one non-diseased site, normalizing it creates a flat line at exactly 1.0. The diseased site may be higher or lower, but very rarely will be exactly 1.0. Therefore, having exactly 1.0 as a result indicates non-diseased
tissue while anything else indicates diseased tissue. With this hypothetical example, that 
algorithm will work almost perfectly at diagnosing non-diseased tissue from diseased 
tissue in spite of the initial spectra before normalization being random with the same 
mean and variance!

Having only and exactly one non-diseased site and one diseased site per patient as 
in the given hypothetical example is an artificial situation. To make it somewhat more 
realistic, the situation can be changed to having two non-diseased sites and two diseased 
sites per patient. Having two non-diseased sites means two spectra have to be averages 
for the mean non-diseased tissue spectrum so neither of the two after normalizing will be 
a line at exactly 1.0. For this new example, assume each spectra has only three emission 
wavelengths with the first assigned to be 460 nm, the second to 600 nm, and the third to 
680 nm. Random spectra will be created with the RANDQ function of Microsoft Excel, 
which is a random number generator which creates values between 0 and 1. Let there be 
created 40 spectra so they can be separated into ten patients with four sites per patient. 
Two sites per "patient" will be arbitrarily assigned to be normal tissue sites and the other 
two sites per "patient" will be assigned to be diseased sites. The graph of the 20 
randomly created spectra assigned to be normal tissue look quite random. (Fig. 4.4) The 
graph of the 20 randomly created spectra assigned to be diseased tissue also look random. 
(Fig. 4.5) Taking the mean and standard deviation has a mean around the range of 0.5, 
varying between about 0.4 and 0.6, as would be expected and with a large standard 
deviation. (Fig. 4.6) When this data was processed with the principal component analysis 
with logistic regression method (PCA/LR) performed with a single data set, a method to
be described in more detail in a later section, the result was 70% sensitivity and 60% specificity. A bit better than 50% for both, but not disturbingly so especially since this was done on one data set and not with a validation set.

Next, the procedure of normalizing to the patient's own non-diseased tissue was performed. Plotting the mean and standard deviation shows that the mean is a flat line at exactly 1.0, as it should be. The standard deviation for the spectra deemed non-diseased tissue has tightened up somewhat when compared to the mean. For the spectra deemed diseased tissue, the mean ended up higher than 1.0 and the standard deviation has gotten worse when compared to the mean. (Fig. 4.7) Using the PCA/LR on the normalized data resulted in 80% sensitivity and 65% specificity, significantly better than the results before normalization. Other runs yielded similar improvements after the normalization, despite the spectra being artificially created with a random number generator. This is because even with more than one non-diseased site per patient, the procedure of normalizing to the patient's own non-diseased tissue itself is still leaking information about what is non-diseased tissue and what is diseased to the routines that follow by making the spectra for the non-diseased tissue group closer to 1.0.

Therefore, the normalization to the patient's own non-diseased tissue can only be done meaningfully if done with strictly a priori knowledge and not by supplying the knowledge of the results the test itself is supposed to find.
4.7.2 Principal Component Analysis

An inverse of a matrix can only be taken if the matrix is square. A matrix inverse can be used to perform operations on matrices akin to division on scalars. If these concepts are unclear, any good book on linear or matrix algebra can provide descriptions of scalars, vectors, matrices, rows and columns, dot products, transposes, identity matrices, and matrix inverses, so these will not be discussed here. [5] The notation used is a bold-font capital letter will represent a two-dimensional matrix and a bold-font lowercase letter will represent a vector.

If the matrix is not square, the pseudo-inverse (also called Moore-Penrose generalized inverse or simply the generalized inverse) can be computed instead and used in much the same way as a standard matrix inverse. [8, 24] Let A be a matrix of size M x N. If M = N, the standard matrix inverse can be calculated. For M > N, the pseudo-inverse of A is computed as followed:

\[ A^* = (A^T A)^{-1} A^T \]  
\[ \text{(12)} \]

For M < N, the pseudo-inverse of A is computed as follows: [5]

\[ A^* = A^T (A A^T)^{-1} \]  
\[ \text{(13)} \]

The superscript \( ^T \) indicates taking the transpose of the matrix and the superscript \( ^{-1} \) indicates taking the inverse of a square matrix. [5, 24, 25] When M > N, multiplying a vector of measured values by the pseudo-inverse creates the least-squares estimate. [5, 24] The equations (12) and (13) above are valid for arrays of real numbers, but to be mathematically precise which can include the case of complex numbers, the formulas above should be changed so the transpose is instead a Hermitian matrix. [24]
A Hermitian matrix is the transpose operation combined with taking a complex conjugate. [26] Since arrays of fluorescence and reflectance spectra contain only real numbers, the transpose of a matrix is equivalent to the Hermitian matrix and the pseudo-inverse formulas given above will work properly.

Although the pseudo-inverse formulas (12) and (13) will work, it does not mean computing the pseudo-inverse is the best way for calculating the least-square estimate. With those formulas, roundoff errors can build up leading to inaccurate results. [24, 25] A better routine to use instead is the singular value decomposition (SVD). [24, 25] The SVD is used for solving most linear least-squares problems. [24, 25] SVD is also the routine usually performed to find the eigendecomposition required to calculate the principal component analysis (PCA). [7, 8]

The SVD has three matrices, U, W, and V, which can be multiplied to form the matrix A with the following formula: [7, 8, 24, 25]

\[
A = U \cdot W \cdot V^T
\]

where:

A: A matrix of size M x N.

U: A matrix of size M x N. It is the column orthogonal matrix; that is, columns are orthonormal. Its columns contains the eigenvectors of the rows of A.

W: A diagonal matrix of size N x N. Contains the singular values (eigenvalues) of A.

V: A matrix of size N x N. An orthogonal matrix which contains in its columns the eigenvectors of the columns of A. V is sometimes called the loading matrix and defines the axis rotations. [7, 25]
Since the columns of \( U \) are orthonormal and \( V \) is an orthonormal matrix, the following rule applies: [25]

\[
U^T \cdot U = V^T \cdot V = 1 \quad (15)
\]

The SVD routine is contained in a built-in function in MATLAB, Student Edition, Version 5.0. This function calculated the SVD for this thesis. The function is written:

\[
[U, W, V] = \text{SVD}(A)
\]

For this thesis, the measured data is a set of fluorescence or reflectance spectra. Background subtraction and calibration of the spectra were performed first. These calibrated spectra are put into matrix \( B \) with the columns of \( B \) being different sites in various patients while the rows of \( B \) represent the different wavelengths measured by the instrument. This step was performed in Microsoft Excel. This matrix was saved as a tab delimited ASCII file. Also saved was a vector listing the patients for each site, \( p \), and another vector listing the pathologist's diagnosis at each site, \( d \). The diagnoses were not used for the PCA step, but only later in the logistic regression step (LR).

The matrix \( B \) and the vectors \( d \) and \( p \) generated as files from Microsoft Excel were then imported into Student Edition MATLAB. If cross-validation was to be performed, a MATLAB program split the matrix into a set of files with a calibration set and validation set for each individual patient. For the \textit{in vitro} bladder analysis, the analysis was first performed with the full set, though, to check for significance there before trying the time-consuming cross-validation analysis. If significant results could
not be obtained with the full set, the results would only get worse after cross-validation because of how it blinds the created diagnostic algorithms to each individual validation set.

Whether the full set was used or cross-validation partitioned the full set into many other sets, before taking the SVD as a step in the PCA method, each spectrum first had to be mean-centered. This mean-centering is the first step for principal component analysis (PCA) and is required so the calculated principal components account for in decreasing order the variance in the data. [7, 8, 9, 13] In this example, each column of B represents a spectrum, so the mean spectrum taken of all the columns of B was subtracted from each column to create a new matrix, A.

PCA takes the set of data in its original coordinate system and puts it in a newly calculated coordinate space. Orthogonal vectors normalized to length one form the orthonormal matrix and provide the basis of the new coordinate space. [7, 8, 9, 27, 28, 29] To understand the value of this new basis system, one should first consider that variation and information content are often strongly related. [7, 29] For PCA, the new basis system is constructed so that the first basis vector is along the line of greatest variation. [7, 8, 11, 29, 30] With the first basis vector established, the second basis vector is found orthogonal to it in the direction of the greatest remaining variance. A third basis vector is then found orthogonal to the first two in the direction of the greatest remaining variance after those two were accounted for. This process continues until the new basis...
system covers the entire space of the original basis. [7, 11, 29] Coordinates in the new basis system are a linear combination of the measured variables in the original basis system. [7, 9]

For the emission spectra in the bladder fluorescence study, the calibrated spectra as measured by the detector at approximately 0.6 nm intervals were linearly interpolated onto a 1 nm increment scale. For the fluorescence spectra in this thesis, the spectral range after this interpolation was from 418 through 700 nm at 1 nm increments. The spectra were then filtered with a five-point moving average filter to reduce the noise. The spectra were then undersampled to reduce the size by two thirds leaving 94 values in each spectra with values spaced every 3 nm. This has the dimension as 94 for the original basis system. Much of the information in those 94 dimensions is highly collinear with little additional information content. PCA projects the set from the original basis space onto a new basis space where each subsequent new orthonormal basis vector accounts for less and less of the variance. [7] Using only a small portion of the new basis vectors taken in order, with decreasing order accounting for less and less variance, will still account for the majority of the variation in the spectra. Using only those new basis vectors which account for the majority of the variation and excluding the rest projects the original data set into a smaller subspace. [7, 9, 29] This causes data compression by leaving in only vectors in the new basis system which significantly influence the measured spectra. The new orthonormal axes removes much redundancy in original data set as shown by the collinearity and the result is a lower-dimensional subspace with little loss in information content. [7, 27]
The lower-dimensional subspace can then be used to reconstruct an estimate of the original measured spectra. The residual error can then be found from the original measured spectra and the estimate of the spectra calculated using the PCA derived subspace. [7, 8]

For the fluorescence spectra of both the bladder *in vivo* and *in vitro* study, the first nine principal components found from PCA were sufficient to provide fits within the noise despite starting with a dimension of 94 values in each spectrum. This demonstrates the great data compression available through use of PCA.

The covariance is taken with respect to more than one source rather than just one. The finding of the sources of variance can be found through the diagonalization of the entire covariance matrix rather than the step-by-step method implied above where each new basis vector is found one at a time in an iterative approach. [28, 29, 30] SVD does the diagonalization of the covariance matrix.

Along with the new basis space, the transformed coordinates of the spectra in the new subspace are needed. In the terminology of PCA, the new coordinates are known as scores. [7, 8] These new coordinates are put into a scores matrix, \( T \), of size \( M \times N \). [7, 8]

\[
T = AV
\]  

(16)

Thus, for the bladder studies in this thesis, taking the scores for the first nine basis vectors stored in \( T \) and multiplying them by the first nine principal component vectors taken from \( V \) reconstructed the spectra to within the noise.
It was mentioned that \( V \) is a matrix of eigenvectors and \( W \) a diagonal matrix of eigenvalues. If we assume \( C \) is an \( N \times N \) square matrix, \( x \) is a non-zero vector of size \( N \times 1 \), and \( v \) is a scalar, then \( v \) is an eigenvalue and \( x \) is an eigenvector if:

\[
C \cdot x = v \cdot x
\]  

(17)

The set of all \( x \) forms a vector space referred to as an eigenspace of \( C \) corresponding to \( v \). [31] The SVD computes these eigenvalues and eigenvectors, which is what was meant by the eigendecomposition.

As was mentioned, the MATLAB function of SVD performed the singular value decompositions for this thesis. Looking at how a singular value decomposition can be performed may help in understanding it. With \( A \) as an \( M \times N \) matrix, let \( D \) be the diagonal matrix with its main diagonal being all the positive singular values of \( A \) and the other elements of the matrix being zero. [24] The \( W \) matrix is created to be the same size as \( A \), an \( M \times N \) matrix, by filling in the remaining values with zero. Thus, \( W \) is square only when \( A \) is square, although \( D \) is by definition square. [24]

\[
A = U \cdot W \cdot V^T = U \begin{bmatrix} D & 0 \\ 0 & 0 \end{bmatrix} V^T
\]  

(18)

The first step is to compute \( A \cdot A^T \), which results in a square matrix, and then compute its eigenvalues and eigenvectors. The second step is with the eigenvalues to construct \( D \). The third step is to have \( V = [V_1 \mid V_2] \) where the columns of \( V_1 \) contain the eigenvectors that correspond to the positive eigenvalues used in \( D \) and \( V_2 \) contains the
rest of the eigenvectors that do not have corresponding positive eigenvalues. The fourth step is to compute \( U_1 = A \cdot V_1 \cdot D^{-1} \), where \( D^{-1} \) is the standard matrix inverse of \( D \). The fifth step is to flesh out \( U_1 \) to be a square matrix by adding elements taken from the identity matrix \( I \). The sixth step is to identify the columns of \( U_1 \) which form the maximal set of linearly independent vectors, then delete the remaining columns and call the trimmed matrix \( U \). [24] Much of this technique is for adapting to \( A \) commonly being a non-square matrix.

While the SVD method of calculating PCA was used for this thesis, another method exists called Nonlinear Iterative Partial Least Squares (NIPALS). While SVD computes the principal components at once, NIPALS replaces that with an iterative approach. [12] With NIPALS, one possible principal component is selected and then adjusted until it converges to the first principal component and then the results from it is extracted from the data set. From the altered data set, another principal component is selected and adjusted until it converges and the results from it are extracted altering the remaining data set still more. As the principal components converge, the PCA and the NIPALS solution become the same so for practical applications either will yield the same results. [12]

Univariate calibration and analysis is the most commonly used method for analyzing spectra. [7] It is easy to use and understand. A single point extracted from each spectra is used for the calibration and analysis. The peak height is often used. There could be errors in that one selected point. It also leaves out much possibly significant spectral information. Theoretically, using multiple points can improve results since more
spectral information is available. Cothren et al. in their colon article extracted normalized emissions at 460, 600, and 680 nm to gain more spectral information since the 460 nm emission was the peak while the ratio of the normalized emission at 680 to 600 nm provided the slope in the red portion of the spectra. [4] Using PCA lets the entire spectrum be used so potentially can find significant values at any emission wavelength. [7, 8]

PCA is one type of multivariate analysis and there are other types too although the other types are not discussed here in detail. [6, 7, 11, 12, 13] Multivariate methods the same as PCA or similar to it have been used by others to analyze fluorescence, Raman, and infra-red (IR) spectra to classify sites or samples into various diagnoses or tissue types. [9, 10, 32, 33]

PCA is essentially the same as Karhunen-Loeve expansion with the spectra, actually discrete measured wavelengths as it comes from the detector, assumed to be effectively continuous. [11, 29, 30] When only certain discrete elements used instead of continuous elements, these are considered factors and this results in describing the procedure as Factor Analysis with PCA being a type of Factor Analysis which uses the entire available spectra. [27, 28, 30, 32] Factor Analysis has been used to processes Fourier Transform-infrared spectra of breast tissue, although that was with certain selected factors rather than the entire spectra. [32]
4.7.3 Logistic Regression

Since our primary aim will be to diagnose non-diseased and diseased tissue, the greatest variation which the basis vectors calculated with PCA in decreasing order may not be the results we are searching for. [7] Instead, the basis vectors with the greatest variation may result from a confounded effect that has little to do with finding the diagnoses of interest. In essence, finding diagnoses is a problem in pattern recognition where there may be other superimposed patterns of little importance for finding the patterns considered important for making diagnoses. [27, 30]

Working with the calibration set, the pathologist's diagnoses of the tissue are now included in the analysis for this logistic regression (LR) step. A least-squares regression of the scores on the dependent variable of the diagnosis determines which of the principal components have significance not simply for accounting for variation in the spectra, but for a model to determine the diagnoses of interest in the study. [7, 8, 9, 13]

Linear regression assumes a continuous relationship while having diagnoses are often dividing into only the two discontinuous states of non-disease and disease. Logistic regression is similar to linear regression, but is developed for discontinuous variables such as these diagnoses. [34, 35]

A logistic function results in a probability between 0 and 1. The formula can be written: [34, 35]

\[
p = \frac{e^{\alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n}}{1 + e^{\alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n}}
\]  

(17)
This formula can be rewritten: [34, 35]

\[
p \left( \frac{1}{1-p} \right) = \left( \frac{e^{\alpha - \beta_1 x_1 - \cdots - \beta_n x_n}}{1 + e^{\alpha - \beta_1 x_1 - \cdots - \beta_n x_n}} \right) \left( \frac{1}{1-p} \right)
\]

\[
= \left( \frac{e^{\alpha - \beta_1 x_1 - \cdots - \beta_n x_n}}{1 + e^{\alpha - \beta_1 x_1 - \cdots - \beta_n x_n}} \right) \left(1 + e^{\alpha - \beta_1 x_1 - \cdots - \beta_n x_n} \right)
\]

therefore:

\[
\left( \frac{p}{1-p} \right) = e^{\alpha - \beta_1 x_1 - \cdots - \beta_n x_n} \quad (18)
\]

then taking the ln of both sides:

\[
\ln \left( \frac{p}{1-p} \right) = \alpha \beta_1 x_1 + \cdots + \beta_n x_n \quad (19)
\]

Equation (19) is an equation that can be used in a linear regression analysis. It still leaves open the question of computing of \( \alpha, \beta_1, \beta_2, \ldots, \beta_n \).

Assign the probability of disease to be \( p \) and the probability for not having disease to be \( (1-p) \). Let \( n \) sites be measured. Let \( y=1 \) if the outcome is diseased and \( y=0 \) if the outcome is non-diseased. Assume there will be \( k \) sites with disease so \( y_j=1 \) for \( j = 0, 1, \ldots, k \), while \( y_j = 0 \) for \( j=k+1, k+2, \ldots, n \). The probability of getting exactly \( k \) diseased sites will be defined as the likelihood function, \( L \), and is calculated with the following formula: [5, 36, 37]

\[
L = p^k (1-p)^{n-k} = \prod_{j=1}^{n} p^{y_j} (1-p)^{1-y_j} \quad (20)
\]

Finding the value of \( p \) that will maximize \( L \) finds the maximum likelihood estimate. [36, 37] Taking the natural logarithm of both sides of equation (20) and then finding \( \ln(L) \) instead does not change the \( p \) value for the maximum likelihood, but
simplifies the calculation. [37] The value for $p$ in equation (20) can then be replaced with how $p$ is defined in terms of $\alpha, \beta_1, \beta_2, ... \beta_n$ in equation (17). After determining the optimum value of $p$, that can be used to in turn find $\alpha, \beta_1, \beta_2, ... \beta_n$.

After running the singular value decomposition in MATLAB and from that calculating the principal components and scores, these were saved as tab delimited ASCII files. These files were imported into the STATA statistical package, Version 5.0. In STATA, the LOGIT function calculated the maximum likelihood estimate of $\ln(L)$ and reported the computed values of $\alpha, \beta_1, \beta_2, ... \beta_n$.

In STATA, the LRTEST function, which stands for the likelihood ratio test, compares the results of a new test with an old test. For the bladder spectra, nine principal components were sufficient to fit the original calibrated spectra to within the noise level. One at a time, each principal component was taken away from the other eight and a test performed with that principal component missing by running the LOGIT function. This was compared to using all nine principal components using the LRTEST function. The LRTEST computed the Probability $> \chi^2$ for comparing two tests. If leaving that principal component out as opposed to having it in caused the Probability $> \chi^2$ to drop below the 0.05 significance level, then the principal component left out was considered diagnostically significant. That principal component was put back in and a different one taken out. Again, the LOGIT function was performed and the result of that was compared with the LRTEST function to the result of including all nine principal components. In this fashion, all nine principal components were tested and the ones that were diagnostically significant were determined.
Once the diagnostically significant principal components were determined, LOGIT was run one more time using only that group of principal components...rather than excluding them as was done to determine which were significant...while all the other principal components were excluded. $\beta_i$ was computed for each diagnostically significant principal component, $i$, determined to be significant as well as an overall $\alpha$.

The results for the validation set were then computed using Microsoft Excel with its matrix manipulation functions. Excel was chosen rather than MATLAB because of its superior graphing capabilities. The nine principal components sufficient for modeling the calibration set were loaded in. So were the calibrated spectra comprising the validation set. The validation set spectra were mean-centered using the previously calculated mean taken from the calibration set. Matrix multiplication was performed between the transpose of the mean-centered validation set spectra and the nine principal components, resulting in the scores matrix for the validation set.

Then, using the $\beta_i$ computed for each principal component, $i$, determined to be diagnostically significant as well as an overall $\alpha$ computed from the calibration set, equation (17) was used to compute the probabilities for every site in the validation set.

A simple decision threshold was to use what had a probability of over 50% as diseased, but that led to high specificity and low sensitivity in cases were there was much more tissue in the calibration set diagnosed as non-diseased than diseased. A weighted decision threshold was computed for the calibration set also:

$$\text{weighted decision threshold} = \frac{\text{number of non-diseased sites}}{\text{total number of sites}}$$ (21)
This weighted decision threshold was then applied to the validation set. It resulted in specificities and sensitivities that were approximately equal.

The decision threshold was also brought from 0.0 to 1.0 in small increments with a sensitivity and specificity at each increment to generate an ROC curve.

4.8 Tissue Model

A study by Richards-Kortum et al. created a model of autofluorescence for detecting atherosclerosis. [38] However, while identifying the individual fluorophores, measuring the light attenuation and scattering, and so forth as done for that study are applicable to mucosal tissues such as bladder tissue, this was a one-layer model. A two-layer model may be needed if the mucosa and submucosa differ significantly in their optical properties.

A study of colon tissue by Zonios et al. created a more sophisticated model with an important part of that model being the fluorescence density functions. These were calculated from carefully oriented fluorescence microscopy images with the vertical axis of the image following what would be the lumen of the colon and the horizontal axis depth into the tissue from the lumen, then integrating in the vertical direction. [39] A goal toward modeling the bladder tissue was to calculated a similar set of fluorescence
density function, f(x). Since the image is made of individual pixels each with amplitude z(x,y) with x being the horizontal direction and y the vertical, integrating along the vertical axis is the equivalent to summing each column.

\[ f(x) = \int_{\text{bottom of image}}^{\text{top of image}} z(x,y) \, dy = \sum_{y=\text{bottom pixel}}^{y=\text{top pixel}} z(x,y) \tag{22} \]

The study by Zonios et al. also used the microscope images to determine the different fluorophores, then thresholding and masking were computed to extract the information from the microscope images for each of the fluorophores of interest. [39] A similar technique was planned for the microscope images of bladder tissue. Assume N different type of fluorophores were found. Then, there will be a different image with amplitudes at each pixel of \( z_i(x,y) \) for the images \( i=1, N \).

\[ f_i(x) = \sum_{y=\text{bottom pixel}}^{y=\text{top pixel}} z_i(x,y) \tag{23} \]
### NOTATION

(A bold-font capital letter indicates a two-dimensional matrix and a bold-font lower case letter indicates a vector)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A matrix of size M x N.</td>
</tr>
<tr>
<td>A</td>
<td>Set of quantities for non-diseased sites.</td>
</tr>
<tr>
<td>B</td>
<td>Set of quantities of diseased sites.</td>
</tr>
<tr>
<td>d</td>
<td>Euclidean distance from point on ROC curve to optimum (0,1) point.</td>
</tr>
<tr>
<td>f(x)</td>
<td>Overall fluorescence density function.</td>
</tr>
<tr>
<td>f_i(x)</td>
<td>Fluorescence density function for fluorophore i.</td>
</tr>
<tr>
<td>F_j(x,y) dx dy</td>
<td>Probability something of category j will have values (x,y).</td>
</tr>
<tr>
<td>FN</td>
<td>False negatives.</td>
</tr>
<tr>
<td>FP</td>
<td>False positives.</td>
</tr>
<tr>
<td>I</td>
<td>General integer counter.</td>
</tr>
<tr>
<td>j</td>
<td>Various categories numbered one on up. In this case, 1 for normal colon tissue, 2 for hyperplastic polyps, and 3 for adenomatous polyps.</td>
</tr>
<tr>
<td>M</td>
<td>Number of rows in A, an integer.</td>
</tr>
<tr>
<td>N</td>
<td>Number of measured wavelengths.</td>
</tr>
<tr>
<td>N_A</td>
<td>Number of non-diseased sites.</td>
</tr>
<tr>
<td>N_B</td>
<td>Number of diseased sites.</td>
</tr>
</tbody>
</table>

Table 4.2: Notation table.
(A bold-font capital letter indicates a two-dimensional matrix and a bold-font lower case letter indicates a vector)

<table>
<thead>
<tr>
<th><strong>Variable</strong></th>
<th><strong>Description</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>p</td>
<td>Probability calculated from logistic function.</td>
</tr>
<tr>
<td>P&lt;sub&gt;j&lt;/sub&gt;</td>
<td>Probability of measured values indicating type j.</td>
</tr>
<tr>
<td>p&lt;sub&gt;j&lt;/sub&gt;</td>
<td>Probability of finding category j in sample population.</td>
</tr>
<tr>
<td>PVN</td>
<td>Predictive value of a negative test.</td>
</tr>
<tr>
<td>PVP</td>
<td>Predictive value of a positive test.</td>
</tr>
<tr>
<td>s&lt;sub&gt;D&lt;/sub&gt;</td>
<td>Pooled variance.</td>
</tr>
<tr>
<td>t</td>
<td>Student's t-test value.</td>
</tr>
<tr>
<td>T</td>
<td>Matrix of size M x N containing the scores after performing PCA.</td>
</tr>
<tr>
<td>TN</td>
<td>True negatives.</td>
</tr>
<tr>
<td>TP</td>
<td>True positives.</td>
</tr>
<tr>
<td>U</td>
<td>A matrix of size M x N. It is the column orthogonal matrix; that is, columns are orthonormal. Its columns contain the eigenvectors of the rows of A.</td>
</tr>
<tr>
<td>W</td>
<td>A diagonal matrix of size N x N. Contains the singular values (eigenvalues) of A.</td>
</tr>
</tbody>
</table>

Table 4.2: Notation table. (cont.)
NOTATION (cont.)

(A bold-font capital letter indicates a two-dimensional matrix and a bold-font lower case letter indicates a vector)

\( V \) A matrix of size \( N \times N \). An orthogonal matrix which contains in its columns the eigenvectors of the columns of \( A \). \( V \) is sometimes called the loading matrix and defines the axis rotations.

\((x, y)\) Measured values.

\( x \) Normalized 680 nm/600 nm in one section. Horizontal location in an image in another section.

\(<x_\lambda>\) Mean of non-diseased sites.

\(<x_\beta>\) Mean of diseased sites.

\( y \) Normalized 460 nm peak. Vertical location in an image in another section.

\( \alpha \) Parameters used in logistic regression analysis.

\( u \) Degrees of freedom.

\( \beta_i \) Parameters used in logistic regression analysis.

Table 4.2. Notation table. (cont.)
REFERENCES


benign mucosal tissue vs. invasive cancer
400 nm excitation

Figure 4.1: ROC curve of benign mucosal tissue vs. invasive cancer after applying the PCA/LR method to the fluorescence measurements at 400 nm excitation.
Figure 4.2: Simple example with two ten-element arrays, one defined as non-diseased and the other as diseased.
Figure 4.3: Two Gaussians created with the standard deviations and means taken from Fig. 4.2.
Figure 4.4: Twenty randomly created spectra with emission values assigned to 460, 600, and 680 nm to be called normal tissue.
Figure 4.5: Twenty randomly created spectra with emission values assigned to 460, 600, and 680 nm to be called diseased tissue.
Figure 4.6: Average and standard deviations for spectra of Fig. 4.4 and Fig. 4.5.
Figure 4.7: Average and standard deviations for spectra of Fig. 4.4 and Fig. 4.5 after the spectra have been normalized to the normal tissue for the assumed patient.
CHAPTER 5

COLON FLUORESCENCE AS TEST OF PRINCIPAL COMPONENT ANALYSIS WITH LOGISTIC REGRESSION METHOD (PCA/LR)

5.1 Objectives

5.1.1 Comparison of PCA/LR Method with Earlier Reported Earlier Reported Bivariate Normal Distribution With Bayes' Theorem Method

A previous joint study with the Cleveland Clinic Foundation and the Massachusetts Institute of Technology produced a diagnostic algorithm that detected dysplasia in colon tissue with a sensitivity of 90% and a specificity of 95% when used in a blinded study. [1] A variety of other studies have also shown fluorescence spectroscopy could detect adenoma in the colon, but it was decided to concentrate on the study by Cothren et al. [1, 2, 3, 4, 5] The methods from this colon tissue study by Cothren et al. were applied to the bladder tissue study which is the main subject of this thesis. [1] However, another algorithm besides the method used in this colon study was developed too for the bladder study. Namely, the method of principal component analysis followed by logistic regression (PCA/LR) as discussed in Chapter Four. Applying PCA/LR on the previous colon fluorescence spectra data appeared to be a method of comparing the two methods to determine if PCA/LR would perform as well as or better than the earlier method.
The method used in the colon study required normalization of the spectra to the patient's own normal tissue at emission wavelengths of 460, 600, and 680 nm. [1] As was discussed at length in the example exercise in Chapter Four, normalizing to the patient's own normal tissue biases the data if the patient's own normal tissue is not known prior to the pathologist's diagnosis. Therefore, the method of this colon study cannot be meaningfully applied to the bladder in vitro study because the disease types of dysplasia and carcinoma-in-situ (CIS) were flat and, to the naked eye of the physician who performed the cystectomy, often indistinguishable from bladder tissue with benign mucosa. For the in vivo bladder study, the greatly predominant disease type was papillary tumor, which was distinguishable by eye from the bladder wall. Dysplasia and CIS were only rarely seen on the flat bladder wall in the in vivo bladder study. Therefore, the normalization procedure used in the colon study might be meaningfully applied for the bladder in vivo study of this thesis and for the polyps of the colon study itself, but not for the in vitro bladder study.

5.1.2 Colon Polyps and Colon Cancer

Early detection of colon polyps by fluorescence spectroscopy before they are visible to the eye through the colonoscope could be useful clinically and save lives. The colon and rectum is the third most common site of carcinoma in men and the second in women. [6] Colorectal cancer is the third most common cause of cancer death according to one source. [6] Other sources state it is the second most common cause of cancer death in the United States. [7, 8] For almost 100% of cases, a biopsy take at colonoscopy
establishes the final diagnosis. [6] With early discovery, colon cancer can be cured by resection. [8] Random biopsies can only sample a very small area of the colon surface, though.

There is evidence that carcinoma in the colon develops mainly from formerly benign adenomatous polyps. [6, 7, 8] There appears to be a chain of progression where normal epithelium forms hyperplastic epithelium which then changes to adenoma. The adenoma can then become dysplasia, then carcinoma-in-situ, and finally invasive cancer. [6, 7, 8] To reduce the risk, any visible adenomatous polyps found during colonoscopy are removed. [6]

Polyps are overgrowths of tissue from mucosal surface which extend into the lumen. [6, 8] The polyps are believed to arise from epithelial cells deep in crypts. [8, 9] Each of these crypts, which are open areas in the mucosal tissue as the name crypt suggests, are lined with columnar epithelial cells. [9] The crypts are surrounded by lamina propria. Approximately 400 μm from the tissue surface is the submucosa for normal colon tissue. (Fig. 5.1) When the epithelial cells inside the crypts undergo abnormal cell proliferation, a polyp forms. [7, 9] These abnormally growing epithelial cells differ from normal epithelial cells in that they are hyperchormatic and lack the ability of secreting mucus. [8]

It has been reported, in part from work I have done in starting this thesis and discussed more in Chapter Ten, that the normal colon epithelial cells do not appreciably fluoresce when excited at 370 nm but the dysplastic epithelial cells do exhibit a broadband fluorescence. [10] In addition, the collagen in the colon tissue fluoresces as
the strongest fluorophore. The colon tissue morphology with the thickness of the lamina propria with its lesser amount of collagen compared to the deeper submucosa with its more densely packed collagen affects the fluorescence spectra measured from the tissue surface. [10] Both the fluorescence of the dysplastic colon epithelium cells and the different tissue morphology of polyps compared to that of normal colon tissue with normal mucosa contribute to the fluorescence amplitude and spectral shape measured from the tissue surface. [10]

Macroscopically, these polyps can be divided into three groups: hyperplastic polyp, which are small, hemispheric, smooth mounds; tubular adenoma, which rise from the colon tissue surface on a stalk; and villous adenoma, which have many papillae giving an appearance similar to shag carpet. [7] Most small hyperplastic polyps have no malignant potential and are not called an adenoma. [7, 8] For Cothren et al., the tissue was therefore broken into three groups, colon tissue with normal mucosa, hyperplastic polyps, and adenomatous polyps. [1] The adenomatous polyps include tubular, villous, and a mixture of both.

Under the microscope, more histologic architecture can be determined. The polyps can be penduculated or sessile, which means on a stalk for the former and flat for the latter. Most tubular adenomas are pedunculated and most villous adenomas are sessile. [6, 7]

For testing the previously reported diagnostic method to compare it with the PCA/LR method, the three categories of normal colon tissue with normal mucosa,
hyperplastic polyps, and adenomatous polyps were used, as well as combining the two types of polyps into a single diseased category so that sensitivity and specificity could be calculated.

5.2 Materials and Methods Specific for Fluorescence Detection at Colonoscopy

5.2.1 Experimental Setup

Tissue was accessed via an optical-fiber probe passed through the instrument channel of a standard colonoscope. This equipment was discussed at length in Chapter Three of this thesis. A total of 57 patients had spectra taken in vivo through the colonoscope. [1] However, for testing PCA/LR method as compared to the earlier diagnostic method of Cothren et al., only a subset of 16 of these patients were used. The fluorescence spectra were taken at 370 nm excitation. [1]

5.2.2 Methods of Analysis

Principal component analysis and logistic regression were discussed earlier in Chapter Four. In this case, nine principal components were calculated for the colon spectra. Then, logistic regression was used and the principal components which passed a pseudo $R^2$ value test at or under 0.05 were used to diagnose the tissue type.

To prevent the problem of biasing the result by testing the data on the same data set that was used to develop the algorithm, an issue discussed in Chapter Four, the data was split into two sets. The first set was tested as a validation set on a diagnostic algorithm developed with the second set. Then, to check against a chance split of the
spectra into sets that gave excellent results that might not hold for other sets, the sets were then switched so that the second set was tested as a validation set on a diagnostic algorithm developed with the first set. In both cases, the validation set spectra were not used to develop the diagnostic algorithm.

The equations for the bivariate normal distributions are taken from the paper by Cothren et al. [1] There are three disease types: normal colon tissue with normal mucosa, hyperplastic polyps, and adenomatous polyps. These are designated in the following equations by the integer variable j with j being 1, 2, or 3 for the three diagnostic tissue types. Let x represent the normalized 680/600 nm values and let y represent the normalized 460 nm value. For each j, a bivariate normal distribution is created. Five parameters define each of these j bivariate normal distributions: $\mu_x$, the mean of the normalized 680/600 nm ratio for the j diagnosis; $\sigma_x$, the standard deviation of the 680/600 nm ratio at the j diagnosis; $\mu_y$, the mean of the normalized 460 nm value at the j diagnosis; $\sigma_y$, the standard deviation of the normalized 460 nm value at the j diagnosis; and $\rho$, the correlation coefficient at the j diagnosis. To simplify the appearance of the following equations, let $\omega_x = x - \mu_x$ and $\omega_y = y - \mu_y$. While calculating the mean and standard deviation is standard for any spreadsheet or mathematical package, the calculation of the correlation coefficient may need to be done more laborious with the following equation:

$$
\rho = \frac{\omega_x \omega_y}{\sqrt{\omega_x^2 \omega_y^2}}
$$

(1)
The bivariate normal distribution function is a function of \( j, x, \) and \( y \), so will be written \( F_j(x,y) \). It is a probability density function, so integrating with respect to all values of \( x \) and \( y \) will result in a value of exactly 1.0.

\[
F_j(x,y) = k \, e^{-\frac{1}{2\sigma_x^2\sigma_y^2(1-\rho^2)} \left[ \frac{\omega_x^2}{\rho^2} - \frac{\omega_y^2}{\rho^2} \right]}
\]

with

\[
k = \frac{1}{2\pi \sigma_x \sigma_y \sqrt{1-\rho^2}}
\]

Using the equations given so far gives the three bivariate normal distributions for the three diagnostic types. The next step is to modify the probabilities using Bayes' Theorem to arrive at \( P_j(x,y) \) using the \textit{a priori} probability assumptions \( p_j \). Cothren et al. used the incidence found in their study as \( p_j \). [1]

\[
P_j(x,y) = \frac{p_j \, F_j(x,y)}{\sum_j p_j \, F_j(x,y)}
\]

The diagnostic decision for which of the \( j \) categories to label the answer was made at each \((x,y)\) by determining which \( P_j(x,y) \) was the largest.
5.3 Results

5.3.1 Bivariate Normal Distribution With Bayes' Theorem Method

Sixteen of the 57 colon patients from a pervious study were reprocessed staring from the raw detector files. [1] The eleven patients that had hyperplastic polyps were reprocessed, then another five patients were reprocessed to bring up the number of adenomatous polyp sites to approximately that of the hyperplastic polyps.

For each patient at each site, there were three reference measurements taken, two background measurements, and seven to twelve tissue fluorescence spectra. For the reprocessing, the two closest reference measurements were averaged and used, the lowest of the two backgrounds was used, and the four most consistent tissue fluorescence spectra were averaged. While the article by Cothren et al. did discuss dividing by references and subtracting backgrounds, which of the multiple spectra at each tissue site and the multiple references and backgrounds were combined, selected, or averaged was not expounded upon. [1] The reprocessed spectra were, as a result, not processed exactly the same way and may produce worse results.

One point of importance for the later bladder analysis in Chapter Seven and Chapter Eight emerged from this reprocessing exercise. One of the expected fluorophores is porphyrin molecules. When excited at 370 nm, it should produce emission peaks at 630 and 680 nm. The hyperplastic polyp spectra with one processed spectrum per site when plotted together in one group for all 16 patients shows one spectrum with an extremely high and sharp 680 nm emission peak with accompanying
lower 630 nm peak, a second spectrum with a moderately high 680 nm emission peak, other spectra with a broad rise in the same area, and many spectra with little rise at in the red portion of the spectra, that extending from 600 nm onward. (Fig. 5.2)

The adenomatous polyp spectra with one processed spectrum per site when plotted together shows one spectrum with a high and sharp 630 nm peak with a lower rise at 680 nm with many other spectra with a broader rise in the red portion of the spectrum and some spectra with little obvious rise in the red. (Fig. 5.3)

Foreshadowing the later chapter on the \textit{in vitro} bladder fluorescence, Chapter Eight, the invasive cancer fluorescence spectra at 370 nm excitation with one spectra per site also shows one spectrum with sharp and high peaks at 630 and 680 nm as would be expected of porphyrin molecules, but the other spectra do not have such sharp, high peaks there. (Fig. 5.4)

The inconsistency of the two large peaks associated with porphyrin molecules in these sets of spectra bore further investigation. Only one fluorescence spectrum at 370 nm excitation was recorded at each site in the bladder, although each was actually forty averaged together immediately by the detector software. The individual spectra that were averaged were not saved separately. In the colon data, in contrast, seven to twelve spectra were recorded at 370 nm excitation at each site.

Plotting the spectra for a single patient at a single site which contained a hyperplastic polyp demonstrates that one of the spectra has much higher values at 630 and 680 nm then the other, even though all the spectra came from the same site. (Fig. 5.5) Also surprising is the spectrum at that site with the large values at 630 and 680 nm is not
the first measured, as might be expected if the porphyrins photobleached, but the fifth.

Plotting the seven spectra for a single patient at a single site containing an adenomatous polyp demonstrates that four of the spectra have large peaks at 630 and 680 nm while the remaining three do not. (Fig. 5.6) This time, the 630 and 680 nm peaks do diminish with time.

Plotting the combined average colon spectra for all sixteen reprocessed patients of the diagnoses of normal colon tissue, hyperplastic polyps, and adenomatous polyps demonstrates a reduction of the overall amplitude with worsening disease. (Fig. 5.7) Moving up and down one standard deviation from the mean for the normal colon tissue doesn’t overlap the peak of the average hyperplastic polyp or of the average adenomatous polyp, which shows clear grouping and suggests diagnostic potential. (Fig. 5.7) Taking the ratio of the average hyperplastic polyp spectrum to that of normal colon tissue produces a curve demonstrating differences in the spectral shapes. The ratio of the average adenomatous polyp spectrum to that of normal colon tissue also demonstrates how its spectral shape differs from that of normal colon tissue. Both ratio plots are relatively flat from 418 nm for the reprocessed spectra, where they start, through about 600 nm, then a dramatic rise in the red area at wavelengths longer than 600 nm. (Fig. 5.8) This result was reported in the Cothren et al. article. [1] The article showed a ratio curve on the graph that climbed to an intensity ratio of about 1.5 whereas the reprocessed spectra ratio only climbs to about 1.0 for adenomatous polyps divided by normal colon
tissue. However, the article used 57 patients rather than just 16, so some discrepancies are expected. [1] Also, which of the seven to twelve spectra at each tissue site may have been used differently.

After normalizing to the patient's own normal tissue for the 16 reprocessed patients, the emission values at 460, 600, and 680 nm were extracted. The ratio of the normalized 680 nm value was taken to that at 600 nm. This was then plotted with the vertical axis the normalized 460 nm values and the horizontal axis the ratio of 680 nm to 600 nm. (Fig. 5.9) This follows the instructions in the article. The three groups for the three diagnoses are visible in this graph as it was for the one in the article, although the article has more normal tissue sites and more adenomatous polyp sites due to having more patients. [1] Following along some more with Cothren et al. article, bivariate normal probability density functions were calculated for each diagnosed group. There were then plotted in a contour graph. (Fig. 5.10) A similar graph was made using the reported values for the bivariate normal distributions in the Cothren et al. article, again keeping in mind the article used more patients. [1] (Fig. 5.11) The contour maps are clearly very similar, except with the results from the 16 reprocessed patients having narrower and longer normal distributions then for the article.

Cothren et al. then applied Bayes' Theorem and found decision lines for diagnosing the tissue into the three diagnostic groups. Unlike many other articles, Cothren et al. had split their total set into two smaller sets, developed the algorithm on one set, and then validated it on the second set. [1] The importance of doing this was discussed in Chapter Four of this thesis.
Cothren et al. reported on their validation set of finding 46 of 48 normal colon tissue sites for 96% detection, 5 of 11 hyperplastic polyps for 45%, and 26 of 29 adenomatous polyps for 90%.

The 16 patients with reprocessed spectra were then divided into two sets with eight patients in each. The plots of the normalized 460 nm emission peak heights vs. the ratio of the normalized value at 680 nm to that at 600 nm for both the first and second sets retain some of the separation for the different diagnoses. (Fig. 5.12 and 5.13) Bivariate normal distributions were then fitted to one set, weighted by Bayes' Theorem, and decision lines were found, as described by Cothren et al. [1]

To ensure that random chance hadn't allowed a particularly fortuitous separation that made the algorithm seem to work better than it would for another random selection, the first set was run as a validation set on a diagnostic algorithm developed on the second set, then the second set was run as a validation set on a different diagnostic algorithm developed on the first set.

With the first set run as validation set on the algorithm developed with the second set, 18 of 18 normal colon sites were found for 100% detection, 5 of 9 hyperplastic for 50%, and 6 of 9 adenomatous polyps for 67%. With the sets reversed, 14 of 15 normal colon tissue sites were found for 93% detection, 6 of 8 hyperplastic for 75%, and 6 of 10 adenomatous polyps for 60%.

The detection for normal colon tissue is extremely good, as it was for Cothren et al., but the detection of adenomatous polyps is much worse and the detection of hyperplastic polyps is somewhat better. A large part of this worsening of detection of
adenomatous polyps while raising the detection of hyperplastic polyps seems to be due to the use of Bayes' Theorem. This theorem was discussed in Chapter Four of this thesis. In short, Bayes' Theorem uses *a priori* distributions to weight the probabilistic answers.

Thus, in the Cothren et al. article, for the set used to develop the algorithm, there were 43 normal colon tissue sites, 8 hyperplastic polyps, and 33 adenomatous polyps in the modeling set. The observed incidence was assumed to be the *a priori* probability. [1] For normal colon tissue, this led to a factor of for \( p_1 = \frac{43}{43+8+33} = 0.51 \), while for hyperplastic polyps it was \( p_2 = 0.10 \), and for adenomatous polyps it was \( p_3 = 0.39 \).

For the first set with eight patients used as the modeling set, \( p_1 = \frac{18}{18+9+9} = 0.50 \), while for hyperplastic polyps it was \( p_2 = \frac{9}{18+9+9} = 0.25 \), and for adenomatous polyps it was \( p_3 = \frac{9}{18+9+9} = 0.25 \). For the second set with eight patients used as the modeling set, \( p_1 = \frac{15}{15+8+10} = 0.45 \), while for hyperplastic polyps it was \( p_2 = \frac{8}{15+8+10} = 0.24 \), and for adenomatous polyps it was \( p_3 = \frac{10}{15+8+10} = 0.30 \). A \( p_2 = 0.25 \) and 0.24 is considerably larger than the 0.10 using the set size from the Cothren et al. article. [1] Even if the bivariate normal distributions for the Cothren et al. article and these reprocessed 16 patients had been exactly identical, this difference in Bayes' Theorem would the result in different answers.

In essence, I did not properly apply Bayes' Theorem as given in the article. The article made an assumption that has some justification that the incidence of disease types seen in the study of 57 patients reflects what would be seen in the patient population in the future. [1] I had instead taken 16 patients as a subset, deliberately taking 11 patients with hyperplastic polyps and thereby changing the relative distribution.
The reason for reporting this at such length is this distinction becomes important for considering in the bladder chapters for *in vivo* and *in vitro* experiments, Chapter Seven and Chapter Eight, where the distribution of papillary tumors ends up extremely different based on the patient selection process. When using Bayes' Theorem, there should be some reason to expect the *a priori* probabilities will be reasonably correct.

Using the bivariate normal distributions, Bayes' Theorem parameters, and resultant decision lines taken directly from the Cothren et al. article for the first set of eight reprocessed patients, 18 of 18 normal colon tissue sites were found for 100% detection, 2 of 9 hyperplastic polyps for 22%, and 9 of 9 adenomatous polyps for 100%. [1] For the second set, 15 of 15 normal colon tissue sites were found for 100% detection, 2 of 8 hyperplastic polyps for 25%, and 10 of 10 adenomatous polyps for 100%. This indicates relative set sizes for the different diagnoses can have a large influence on the final results. This issue will be discussed more in the bladder chapters, Chapter Seven and Chapter Eight.

If there are only two categories of non-diseased and diseased, then the results can be expressed in terms of sensitivity and specificity. The can be done by considering both hyperplastic polyps and adenomatous polyps as the diseased category and normal colon tissue as the non-diseased category. Using the method of the article except with two bivariate normal distributions rather than three, validation the first set of eight reprocessed patients on an algorithm developed with the second set, the result was 83% sensitivity and 94% specificity. Reversing the sets resulted in a 94% sensitivity and 80% specificity.
For the sets in the article, the reported results were 90% sensitivity and 95% specificity, which is better but the results from the 16 reprocessing patients broken into two sets are still good too. [1]

5.3.2 PCA/LR Method

Since the previous method used data normalized to the patient's own normal tissue which causes the patient's normal tissue to act as the spectral calibration, this same normalization was done before performing PCA/LR so the results could be more easily compared.

With my first set run as validation on my PCA/LR algorithm developed with the second set, 14 of 18 normal mucosal tissue sites were found for 78% detection, 3 of 9 hyperplastic for 33%, and 8 of 9 adenomatous polyps for 89%. The first four principal components were all found significant under 0.05 through the logistic regression step. The first principal component is predominantly flat and would seem mainly to represent overall amplitude. (Fig. 5.14) The second principal component has a large red rise starting just before 600 nm. These suggest that the method of the Cothren et al. article using the extracted wavelengths of 460, 600, and 680 nm with the 460 nm for the overall amplitude and the ratio of 680 nm/600 nm for the slope of the red rise were good choices. [1] The third principal component has a broad dip from about 550 through 600 nm which could possibly be from the 580 nm absorption associated with hemoglobin. The largest feature in the fourth principal component is a dip at 420 nm, which is likely the Soret hemoglobin absorption band.
To insure that the random selection did not by luck produce sets that worked extraordinarily well, the sets were then switched so the second set was run as validation on a PCA/LR algorithm developed with the first set. With this, 13 of 15 normal mucosal tissue sites for 87% detection, 7 of 8 hyperplastic for 88%, and 9 of 10 adenomatous polyps for 90%.

With the second set as the validation set, the algorithm would appear to work much better for detecting hyperplasia then when the sets are used in the opposite order. This could be a problem due to the small number of samples in each set, especially for hyperplastic polyps.

One method for dealing with the small sets is to put hyperplastic and adenomatous polyps together into a single disease category. With my first set as validation on my PCA/LR algorithm developed with the second set, I get a 100% sensitivity and 87% specificity. When the sets are flipped, I get a 94% sensitivity and 94% specificity.

5.4 Discussion

Since the published algorithm for diagnosing the colon spectra depends on, after normalizing to the patient's own normal tissue, the ratio of 680 nm emission to that at 600 nm, the great variation in the sharp and tall 630 and 680 nm peaks seen in the individual spectral plots is of some concern for both the colon and bladder. The height and sharpness of the peaks make it likely porphyrin molecules are the true cause. Richards-Kortum attributes the emission fluorescence in the red part of the spectra that seems to be from porphyrins to porphyrins in bacteria, citing an earlier study that found a
lack of red fluorescence in culture for ulcerated squamous cell carcinoma. [11] While this study here of colon tissue fluorescence does not establish the answer, the variability in the height and that the distinctive peaks disappearance do not seem to be a result of simple photobleaching. (Fig. 5.5) A possible reason is the porphyrins may be highly localized with the probe sometimes on the small area and sometimes not.

The error of taking a subset of 16 patients for the colon spectra which had a different relatives sizes of the three different diagnoses and expecting, but not getting about the same results as when the entire data set of all 57 patients illustrated the importance of relative set sizes. This came into play later when working with the bladder in vitro analysis where the set sizes of dysplasia, CIS, and papillary tumors were very small compared to that of normal mucosal tissue. How this was dealt with there is discussed in Chapter Eight, but this reanalysis of a portion of the colon spectra warned that this would need further examination.

For the set of 16 colon patients with reprocessed spectra when processed with two categories of non-diseased and diseased, the PCA/LR algorithm did a little better than the reported method by Cothren et al. when applied to the same set. [1] The fitted bivariate normal distributions weighted with Bayes' Theorem produced an 83% sensitivity and 94% specificity when validated with the first set of eight patients and a 94% sensitivity and 80% specificity when validated with the second set of eight patients. In contrast, the PCA/LR method had a 100% sensitivity and 87% specificity and then a 94% sensitivity and 94% specificity, respectively. On the other hand, the PCA/LR method also indicated by the shape of the diagnostically significant principal components that using the
normalized 460 nm peak for the overall amplitude, similar to what the first principal component determined, and the ratio of the normalized 680 nm to the 600 nm values for the slope of the red rise of the spectrum, which was similar to what the second principal component determined with its ramp upward in the red portion of the spectrum.

For the bladder in vivo cystoscopy study discussed in its own chapter, Chapter Seven, a similar problem occurred as seen in this test case with 16 colon patients as discussed earlier with the PCA/LR method for the three different diagnoses. That is, after splitting the full set into two roughly even sets, excellent diagnostic results were calculated when one set was used as the modeling set for developing the algorithm and the other set was used as the validation set, but not such excellent results were produced when the role of the sets was reversed. For the in vivo bladder chapter, Chapter Seven, a time consuming method of leave-one-patient-out cross-validation compensated for the small set size. This cross-validation technique is discussed in more Chapter Four. As the reprocessed spectra for 16 colon patients was mainly to serve as a test of PCA/LR method compared to an earlier diagnostic method, the cross-validation technique was not performed on the colon data.
REFERENCES


Figure 5.1: Hematoxylin and eosin stained microscope slide of colon tissue showing cross-section from lumen into tissue along horizontal axis.
Figure 5.2: Colon hyperplastic polyp fluorescence spectra, one spectra at each site, taken at 370 nm excitation.
Figure 5.3: Colon adenomatous polyp fluorescence spectra, one spectra at each site, taken at 370 nm excitation.
Figure 5.4: Bladder invasive cancer fluorescence spectra taken at 370 nm excitation.
Figure 5.5: Colon hyperplastic polyp fluorescence spectra with several spectra taken only at one site in a single patient.
Figure 5.6: Colon adenomatous polyp fluorescence spectra with several spectra taken only at one site in a single patient.
Figure 5.7: Average fluorescence spectra at 370 nm excitation for colon tissue with diagnoses of normal tissue, hyperplastic polyps, and adenomatous polyps along with lines showing plus and minus one standard deviation from mean for average of normal tissue.
Figure 5.8: Ratio of the mean fluorescence spectra at 370 nm excitation for hyperplastic polyps and adenomatous polyps divided by that of normal colon tissue.
Figure 5.9: Diagnostic parameters for entire set of selected 16 patients with reprocessed spectra. The vertical axis labeled 460 N is the 460 nm emission peak intensity of the spectra normalized to the patient’s own normal tissue. The horizontal axis labeled 600 N/680 N is the normalized 600 nm divided by 680 nm intensity ratio.
Figure 5.10: Bivariate normal probability density functions calculated from entire set of selected 16 patients with reprocessed spectra.
Figure 5.11: Bivariate normal probability density function for modeling set of 22 patients in modeling set from article by Cothren et al. [1]
Figure 5.12: Diagnostic parameters for randomized eight patients out of 16 patients with reprocessed spectra to form first data set.
Figure 5.13: Diagnostic parameters for eight patients out of 16 patients with reprocessed spectra not used in first set to form second data set.
Figure 5.14: Four diagnostically significant principal components calculated from the second set for developing a diagnostic algorithm to testing on the first set.
6.1 Overview

This chapter examines bladder anatomy, bladder tumors, and other bladder diseases such as proliferative cystitis, which could be mistaken for a bladder tumor.

A new classification system came out very recently from the World Health Organization and International Society of Urological Pathology (WHO/ISPU) in December of 1998 and will be discussed in this chapter. [1]

6.2 Gross Anatomy of the Urinary Bladder

The urinary bladder stores urine temporarily before the urine is excreted from the body. The bladder holds about 800 ml of urine when under voluntary control and it holds 2000 ml if the connection to the spinal cord is severed. It has smooth muscle in the wall to expel the contents. When empty, the bladder deflates like a balloon. The wall of the empty bladder folds into rugae. [2]

The activation of the detrusor muscles causes the urinary bladder to be voided. The closure of the bladder neck uses fibroelastic tissue, so is closed when the detrusor muscle is relaxed. [3]
The superior direction indicates toward the head and inferior toward the feet. The anterior is toward the front of the body and posterior toward the back. During filling, part of the superior and inferior walls form lateral walls.

An area at the floor of the bladder is called the trigone. It is flattened and triangular in shape. The triangle’s corners are formed from three openings, two are for the ureters and one is for urethra. Flaps near the ureteral openings act as one-way valves to prevent reflux of urine to the kidneys.

The bladder is extraperitoneal; that is, other than the top or superior portion, it is not covered by the peritoneum. The fundus, or trigone, changes little in its location in the body as the bladder fills. The apex of the bladder, though, lifts from the pelvis. This results in a space forming in front of the unperitonealized anterior portion of the bladder.

In the embryo, the urachus forms a tract from the bladder to the umbilicus. Rather than becoming closed off, the urachus can sometimes remain partly or completely open. When this happens, it can collect urine, which can lead to infection. The urachus can also form urachal cysts. These cysts may be associated in some cases with the development of adenocarcinoma.

Inferior to the bladder for males but not females is the prostate gland. The prostate provides fluid during ejaculation. The urethra, which is the exit for urine from the urinary bladder, consists of three parts: the prostatic urethra, the membranous urethra, and the spongy urethra. Besides the urethra, there are also two ureters, which open into the bladder. These ureters connect to the kidneys.
6.3 Anatomy of the Bladder Wall

The wall of the bladder, moving from the lumen outward, consists of epithelium (also called urothelium), lamina propria, and perivesical fat. [4, 5, 6] (See Table 6.1) The wall contains both blood and lymph vessels. [4]

The urothelium is a uniform transitional epithelium underneath which is a thin basal lamina. The topmost single layer of cells of the urothelium is called the superficial zone and consists of large flattened cells, called umbrella cells, which cover relatively large areas. [5] WHO/ISUP prefer the term urothelium for the epithelial cell layer of the bladder, but transitional is still allowable as a synonym. [1]

The urinary bladder actually has two muscle layers. The first is the muscularis mucosae, which is a rarely complete or usually intermittent thin layer of smooth muscle. Next is the muscularis propria, which is called the true muscle. [7] It is important to know whether a tumor has invaded merely the muscularis mucosae or instead the muscularis propria to give a correct description of the stage of the tumor.
1. Urothelium
   This is the epithelial lining composed of transitional epithelium. As the bladder stretches, the epithelium flattens. The epithelium protects the bladder from hypertonic urine.

2. Lamina Propria
   This is fibrous connective tissue

3. Detrusor muscle
   This imparts pressure to bladder contents. It has fibers at organized at right angles in several layers.

4. Serosa
   This is part of the peritoneal lining of the abdominal cavity and is only on superior surface of bladder.

Table 6.1: Four layers of the bladder wall. [2, 6]

Urothelium can be further subdivided into three zones. The first and innermost is the superficial zone. It consists of large flattened cells, called umbrella cells, which cover relatively large areas. The next zone is the intermediate zone. Its thickness varies depending on if the bladder is distended or contracted. It is four-to-five cell layers thick when the bladder is distended and six-to-eight when contracted. The third zone is the basal zone, which consists of only a single layer of small cells. These cells flatten in the stretched bladder but are cylindrical in the contracted bladder. [5]

6.4 Anatomy of the Bladder in Regard to Females and Males

Females are eight times more prone to bacterial infection of the urinary bladder than males. [5] One reason for this is that the urethra is much shorter in the female than
the male, so bacteria have a shorter route from outside the body to the bladder itself. [2, 5, 8] A second reason is that females do not have a prostate gland and the prostate gland produces an antibacterial fluid. A third reason is that females undergo hormonal changes that appear to affect the adherence of bacteria to the urethral wall. A fourth reason is that the urethra in the female is more likely to undergo trauma during sexual intercourse than the urethra in the male. [8]

For older males, enlargement of the prostate gland is common and this can obstruct the urinary tract, making voiding of the bladder difficult. [5] Older females can have prolapse of the uterus, which can pull with it the floor of bladder such that the bladder protrudes into the vagina. When this happens, a pouch forms called a cystocele that fails to empty during urination. [5]

Perhaps because of these anatomical differences between the sexes, 75% of bladder cancer occurs in males. [9] Females have one-third as many transitional cell tumors as males and two-thirds as many squamous cell tumors. [5]

6.5 Bacterial Infection

For bacteria to reach the bladder, there must almost always be an ascending infection. Colonization of bacteria is affected by adhesion to urothelial cells. The receptors on the urothelial cells that affect adhesion of bacteria are "identical to the gycosphingolipids of the P blood group system." [8]
Urinary tract infections (UTI) can have involvement of bladder and/or the kidneys. Vesicoureteral reflux (VUR) is a condition where urine travels up one or both ureters during urination. This can expose the kidneys to bacteria. VUR can be seen with radiopaque dye when voiding. [8]

When there is urine in stasis, the bacteria in it can continue to multiply without being flushed out. [8] This is why a cystocele or open urachrus increase the risk of bacterial infection.

The antibacterial mechanism that attempts to prevent infection is still poorly understood. [8]

6.6 Cystitis

Cystitis relates to acute or chronic inflammation of the urinary bladder. There are various types of cystitis including: hemorrhagic cystitis, which has blood in the tissue; suppurative cystitis, which has a suppurative exudate; ulcerative cystitis; and polypoid cystitis. Cystitis occurs more often in young women of reproductive age and in the elderly of both sexes. [5]

The type of bacteria called coliforms including Escherichia coli, Proteus, Klebsiella, and Enterobacter species can all cause cystitis. Not just bacteria, but viruses such as the adenovirus can also cause cystitis. Schistosomiasis, which is infection with schistosoma hematobium, a type of blood fluke, can also cause cystitis. Schistosomiasis is rarely seen in the U.S., but is seen in countries such as Egypt. [5]
Chronic cystitis can lead to fibrous thickening in the tunica propria, which can make the bladder wall thick and inelastic. [5]

6.7 Diverticulum

A diverticulum is a pouch or sac in the wall of the bladder. It can be either a congenital or an acquired lesion resulting from persistent urethral obstruction, with the latter more likely. The latter is cause in older men most often by an enlarged prostate blocking the outflow of urine. A diverticulum predisposes the bladder to infection because urine can collect in it and remain stagnant, which allows bacterial growth. A diverticulum also predisposes the bladder to from calculi. [5]

Exstrophy is a developmental failure such that the anterior wall of the bladder either communicates directly through a large defect with the anterior abdominal wall or lies as an opened sac. This leads to chronic infections, epithelial metaplasia, and cancer. [5]

6.8 Reactive Changes in Bladder Tissue

Various insults can cause reactive changes in bladder tissue. Inflammation can cause multinucleation, increased nuclear size, chromatin coarseness, prominent nucleoli, and mitotic activity. Distinguishing between the cellular changes resulting from tumors compared to inflammation is important to prevent misdiagnosis. Unlike bladder cancer,
for inflammation there is little change in the following: cell uniformity within groups, central nuclear placement, N:C ratio (nucleus to cytoplasm ratio), cytoplasmic vacuoles, smooth nuclear contours, and evenly dispersed chromatin. [7]

Both radiation treatment and chemotherapy can result in further reactive changes in bladder tissue. For pathologic or cytologic tests, these reactive changes should be watched for and not interpreted as further cancer. Chemotherapy can cause vacuolization; round nuclei; granular, evenly dispersed, peripherally condensed chromatin; and degenerative changes such as irregular nuclei with homogeneous, opaque chromatin, pyknosis (reduction in size of the cell or nucleus), and karyorrhexis (fragmentation of nucleus). Radiation causes an increase in cell size but preserves the N:C ratio. Radiation also results in multinucleation, nuclear vacuolization, and cytoplasmic vacuolization. [7]

6.9 Neoplastic Tumors

The word tumor literally means swelling, but most current usage instead means almost exclusively neoplastic masses, not nonneoplastic. The word neoplasm means new growth, and the origin of all neoplasms is loss of responsiveness to normal growth controls. Neoplasms behave as parasites, invade, spread, and compete with normal tissue for metabolic needs. [10] Tumor invasion means that the tumor has penetrated through the lamina propria. [5, 7]
6.10 Morphologic Patterns of Bladder Cancer

There are four morphologic patterns of bladder tumors: papilloma-papillary carcinoma; invasive papillary carcinoma; flat noninvasive carcinoma; and flat invasive carcinoma. [5, 11]

Ro et al. use three groups of TCC: superficial papillary, carcinoma in situ (CIS or TIS), and invasive. Invasive tumors are those that cross the lamina propria. This would roughly correspond to papilloma-papillary carcinoma, flat noninvasive carcinoma, and flat invasive carcinoma mentioned above. In Ro et al., it is also mentioned that a low grade papillary tumor can have an infiltrating tumor at its base, which would be about the same as invasive papillary carcinoma. [7]

A papilloma is a solitary benign papillary tumor with no more than eight cell layers of normal-appearing transitional epithelium on a fibrovascular core. [7, 12] WHO/ISPUP note that because of possible tangential sectioning, counting cell layers may not be meaningful. [1] Papillary tumors are tumors that have finger like fronds projecting from the surface of a tissue. [10] It can be hard to distinguish papilloma from grade I papillary carcinoma. [1, 5, 12]

6.11 Staging of Primary Tumor for the Urinary Bladder

The stage of a bladder tumor is determined by looking at its depth of penetration. (See Table 6.2)
<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
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<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Ta</td>
<td>Papillary non-invasive or papilloma</td>
</tr>
<tr>
<td>Tis</td>
<td>In situ: &quot;flat tumor&quot;</td>
</tr>
<tr>
<td>T1</td>
<td>Subepithelial connective tissue</td>
</tr>
<tr>
<td>T2</td>
<td>Superficial muscle (inner half)</td>
</tr>
<tr>
<td>T3</td>
<td>Deep muscle or perivesical fat</td>
</tr>
<tr>
<td>T4</td>
<td>Prostate, uterus, vagina, pelvic wall, abdominal wall</td>
</tr>
</tbody>
</table>

Table 6.2: Staging of bladder cancer. [13]

The "1" though "4" indicate depth of penetration for an invasive tumor. "T" indicates the evaluation of a primary tumor. "a" indicates a papillary non-invasive tumor.

These stages can be further subdivided. T3 can be described as either T3a or T3b where T3a indicates deep muscle invasion and T3b indicates invasion into the perivesical fat. Likewise, T4 can be subdivided into T4a which is invasion of the prostate, uterus, or vagina and T4b which means that the tumor has reached the pelvic wall or abdominal wall. [13, 14]

Metastatic spread of the tumor also has a classification system. (See Table 6.3)
N0: No lymph node involvement
N1: Single, homolateral lymph node involvement
N2: Contralateral, bilateral, or multi-lymph node involvement
N3: Fixed pelvic wall mass separate from primary tumor
N4: Juxtaregional lymph node involvement
M: Distant metastasis

Table 6.3: Metastatic classification. [14]

Although not tumors themselves, hyperplasia and dysplasia have been suspected of being antecedents to the development of neoplasms. [5] However, a new classification by WHO/ISUP claims hyperplasia does not have premalignant potential, while dysplasia may be treated as having it. [1] That source also notes great variability in what pathologists call dysplasia. [1] Hyperplasia is a thickening of mucosal layer due to increased number of cells, whereas dysplasia is an atypical hyperplasia including nuclear atypia. [1, 5]
Normal
   Normal (may include former "mild dysplasia")
Hyperplasia
   Flat hyperplasia
   Papillary hyperplasia
Flat lesions with atypia
   Reactive (inflammatory) atypia
   Atypia of unknown significance
Dysplasia (low-grade intraurothelial neoplasia)
   Carcinoma in situ (CIS) (includes "severe dysplasia")
Papillary neoplasms
   Papilloma
   Inverted papilloma
   Papillary neoplasm of low malignant potential
   Papillary carcinoma, low-grade
   Papillary carcinoma, high-grade
Invasive neoplasms
   Lamina propria invasion
   Muscularis propria (detrusor muscle) invasion

Table 6.4: The WHO/ISUP consensus classification. [1, Table 1, p. 1436]

For the studies of this thesis, the diagnoses of benign mucosal tissue (what Table 6.4 refers to as normal), dysplasia, CIS, invasive neoplasms, and papillary tumors were used.

For completeness sake, there is an older system used to designate the stage of bladder cancer uses the letters A, B, C, and D instead of T. (See Table 6.5)
Table 6.5: Another system for staging bladder cancer. [5]

The stage of the tumor determines the prognosis for recurrence and survival. (See Table 6.6) Less than 10% of patients with papillomas develop invasive carcinoma so most won't influence survival. [5]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival by Stage at Diagnosis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ta, CIS, T1</td>
</tr>
<tr>
<td>Transurethral resection</td>
<td>47-81</td>
</tr>
<tr>
<td>Segmental resection</td>
<td>43-100</td>
</tr>
<tr>
<td>Simple cystectomy</td>
<td>27-88</td>
</tr>
<tr>
<td>Radical cystectomy</td>
<td>63-83</td>
</tr>
</tbody>
</table>

Table 6.6: Five-year survival rates after bladder cancer surgery. [14, Table 3, p. 923]

However, there is much controversy over the distinguishing papillomas from low grade papillary carcinoma. [1, 5, 12] For the different types of papillary tumors, a new classification system from December 1998 has a category of Papillomas which are strictly benign; a category of Papillary Urothelial Neoplasm of Low Malignant Potential which can lead to invasion in only very rare cases; a category called Papillary Urothelial

0: limited to mucosa
A: Invades lamina propria but not muscularis
B₁: Invades superficial muscle layer
B₂: Invades deep muscle
C: Invades perivesical region
D₁: Exhibits regional metastases
D₂: Exhibits distant metastases
Carcinoma, Low Grade which has less than a five percent chance of leading to invasion and which may have only subtle changes from the previous category; and a category called Papillary Urothelial Carcinoma, High Grade which has a 15% to 40% chance of leading to invasion. [1] None of these papillary tumors have any certainty of progressing to invasion.

6.12 Tumor Grade

The grade of a tumor is based on the amount of atypia. Bladder tumors are usually stated as having grades I, II, and III. [5] Grade I tumors are almost always papillary. Grade II tumors are often papillary, but are sometimes flat or invasive instead. Grade III tumors are mostly flat or invasive, but some are still papillary. [5, 7, 11] (See Table 6.7)

Grade II cancers are a heterogenous subgroup, so they are often subdivided to IIa and IIb using nuclear pleomorphism and number of mitoses to make the division. [7]
<table>
<thead>
<tr>
<th>Observation</th>
<th>Papilloma</th>
<th>TCC-I</th>
<th>TCC-II</th>
<th>TCC-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplasia</td>
<td>None</td>
<td>Variable</td>
<td>Variable</td>
<td>Prominent</td>
</tr>
<tr>
<td>Superficial cell layer</td>
<td>Preserved</td>
<td>Variable</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>&quot;Clear&quot; cytoplasm</td>
<td>Present</td>
<td>Often</td>
<td>Often</td>
<td>Absent;</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>Absent</td>
<td>vacuoles</td>
<td>common</td>
</tr>
<tr>
<td>Pleomorphism</td>
<td>None</td>
<td>Variable</td>
<td>Variable</td>
<td>Prominent</td>
</tr>
<tr>
<td>Nuclear polarization</td>
<td>Normal</td>
<td>Slightly</td>
<td>Abnormal</td>
<td>Absent</td>
</tr>
<tr>
<td>Nuclear polarization</td>
<td>None</td>
<td>Slight</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Nuclear crowding</td>
<td>Normal</td>
<td>Fine-regular</td>
<td>Fine-regular</td>
<td>Course-regular</td>
</tr>
<tr>
<td>Chromatin</td>
<td>Normal</td>
<td>Fine-regular</td>
<td>Fine-regular</td>
<td>Course-regular</td>
</tr>
<tr>
<td>Mitoses</td>
<td>Rare</td>
<td>Uncommon</td>
<td>Common</td>
<td>Prominent</td>
</tr>
</tbody>
</table>

Table 6.7: Classification of bladder tumors.
[5, Table 22-2, p. 1091; 9, Table 2, p. 18]

6.13 Clinical Symptoms of Bladder Cancer

The most common and often the only clinical symptom of bladder cancer is hematuria, or blood in the urine. This can be painless. [11] Another clinical symptom is frequent urination. Urgency of urination is another. Dysuria, or pain during urination, is sometimes a clinical symptom of bladder cancer. [5]

6.14 Cystoscopic Examination

A cystoscope is an endoscope used to visually inspect the lumen of the bladder to look for abnormalities and growths. Biopsy specimens can be acquired using the cystoscope. However, early invasive carcinoma, carcinoma in situ, and dysplasia can all
be difficult to detect with a cystoscopic examination because they can lie flat on the tissue. Sloughing and loss of the epithelium in a biopsy section may prevent diagnosis by the pathologist. [7]

6.15 Urologic Cytology

Urologic cytology is the examination of exfoliated cells within urine. It has the advantage that cells may be shed from flat situ lesions that cannot be readily seen through a cystoscope. A major disadvantage is that generally there is a low cellular yield in the urine. Reactive atypia in the bladder from inflammation or other causes can be misdiagnosed as a tumor because some of the reactive changes look similar to those changes in tumor cells. Cytology also has trouble identifying grade 1 TCC because the changes in the cells in grade 1 are not easily recognized. [7]

Urologic cytology works well as an additional test along with using a cystoscope. Since, as was mentioned earlier, most grade I bladder tumors are papillary tumors, the papillary fronds are usually visible from the endoscope even though these papillary tumors are often missed in the cytologic test. On the other hand, the cytologic test may reveal flat tumors that are invisible to the endoscope. [7]

For a cytologic test using a bladder washing, the test may miss ureteral, urethral, or renal pelvic tumors. [7]

The cytologic samples are examined for the following list of malignancy identifiers: increased cell and nuclear size, increased nuclear-cytoplasmic (N:C) ratio, markedly eccentric positioning of the nucleus, nuclear pleomorphism and irregularity,
hyperchromasia, chromatin clumping, nuclear crowding, prominent nucleoli, mitotic figures, lack of cytoplasmic vacuolization, loss of cell cohesion, and the presence of papillary formation. [7]

There are several problems which make it difficult to properly recognize the correct malignancy indicators. The cells from the bladder include a variety of cell types. Besides the normal urothelial cells, there is a top layer of umbrella cells, which are enlarged and multinucleated, but have a normal N:C ratio. The basal cells and the cells of the epithelium of the ureter and renal pelvis are cuboidal, and the cuboidal shape gives the illusion of an increased N:C ratio. Massage of the prostate can release seminal vesicle cells, which have enlarged, hyperchromatic polypoid nuclei and pigmented cytoplasm. Besides the different cell types, various insults to the bladder can cause nuclear and/or cytoplasmic reactive changes without a tumor being present. Some of these insults include viral infection, lead poisoning, and fulguration of earlier tumors. [7]

Abnormalities may not be found in every voided specimen because bladder-cancer-induced hematuria may be intermittent. Also, the number of red blood cells (RBCs) in the urine only roughly correlates with tumor grade, stage, or size. For example, physical exertion or sexual activity can greatly increase number of RBCs excreted. Another problem is that the RBCs can lyse if the urine has a specific gravity less than or equal to 1.008. [15]

There are many suggestions for getting good urine samples for a cytological test. The first specimens voided in the morning shouldn't be used because the urine has been
stagnant all night, which could allow bacteria growth. For similar reasons, 24-hour collections and urine from drainage bags should be avoided. Having the patient take 1 gram of vitamin C will create a more optimal pH of 5 in the urine. [7]

### 6.16 Other Tests for Detecting Bladder Cancer

Various other tests besides urologic cytology and the cystoscopic examination exist or are being developed. The following factors are thought to affect tumor growth: grade, cell type, TNM stage, tumor growth pattern, size of tumors, number of tumors, blood vessel invasion, lymphatic invasion, site of tumor, nuclear shape in tumor cells, karyotype (the chromosomal constituents of the cell nucleus), type of infiltration, antigenic phenotype, growth factors/receptors, and oncogene expression. [14] This is a long list and different tests are being developed or have been developed to try and measure some of these factors.

Some of the tests being tried are immunostaining for laminin and type IV collagen to check for basement membrane integrity; human milk fat globulin-2 (HMFG-2) staining; testing for monoclonal antibody 32-2B to desmosomal glycoprotein to detect stromal invasion; and checking for transferrin receptor positivity. [7]

Various DNA tests also seem promising by looking for abnormalities in chromosomes 1, 5, 7, and 9. Finding the c-erb-B oncogene located on chromosome 7 seems significant. [7] The c-myc gene product may be an indicator of tumor grade.
Fluorescent in situ hybridization (FISH) uses fluorescent probes and nonisotopic detection procedures to detect chromosome abnormalities. A high S-phase fraction also indicates tumors that are often progressive. [7]

Microscopic urinalysis is being compared with commercially available chemical reagent strips. The strips work even when the RBCs lyse. Although urine analysis has the problems discussed earlier such as low cellular yield, these reagent strips may allow patients to test themselves at home so that bladder cancer, especially flat tumors, might be detected earlier. [15]

**6.17 Bladder Cancer in Newly Presenting Patients**

Newly presenting patients with bladder cancer have only superficial tumors (Ta, CIS, or T1) 75% of the time. 20% of the time, newly presenting patients have deep tumors (T2, T3, or T4). 5% of the time, the tumors are already metastatic (N or M). [14]

**6.18 Types of Bladder Cancer**

The World Health Organization (WHO) recognizes four primary histologic types of bladder cancer: TCC, squamous cell carcinoma, adenocarcinoma, and undifferentiated carcinoma. [7] (See Table 6.8) In addition, there are rare occurrences of sarcomatoid carcinoma, small-cell carcinoma, and lymphoepithelioma. [7]
1. Transitional-cell carcinoma (TCC) (90% of cases in the U.S.)
   A. Superficial papillary
   B. Carcinoma in situ (CIS or TIS)
   C. Invasive
2. Squamous-cell carcinoma (6-7% in the U.S.)
3. Adenocarcinoma (1-2% in the U.S.)
4. Undifferentiated carcinoma (<1% in the U.S.)

Table 6.8: WHO's histologic types of bladder cancer. [7]

The most commonly occurring type of bladder cancer is TCC. TCC can be judged on three scales: from papillary to flat, from noninvasive to invasive, and from well-differentiated to highly anaplastic. [5, 11]

For the purpose of the autofluorescent bladder study being done here at the Cleveland Clinic, I think it may be beneficial to initially keep the flat tumors and the papillary tumors in separate groups. The infiltration pattern is different. The infiltration of a flat tumor is described as "tentacular," whereas the infiltration of a papillary tumor is described as "broad front." [16] Furthermore, when the papillary tumor invades, for grade 1, the urothelium is normal or near normal in thickness; for grade 2, the urothelium is thickened; and for grade 3, the urothelium can be either thickened or denuded. Flat carcinoma in situ, in contrast, does not seem to get a thicker urothelium at all, but instead erodes and then denudes. [6]

6.19 Superficial Tumors

Superficial tumors are either confined to the mucosa (stage 0, pTa) or they are confined to the lamina propria (stage A; pT1). [7] One study by Greene of patients who
had papillary tumors found that after following for 15 years or more, 73 percent had recurring tumors, 22 percent had recurrent neoplasms of grade 2 or higher, and 10 percent had invasive cancer. [12] Another study by Althausen found that for patients with non-invasive papillary carcinoma, 30 percent developed invasive cancer. [17] Ro et al. found only one third of superficial bladder tumors become invasive with time. [7] The desire to know which third will become invasive has lead to a search for a method for determining whether superficial tumors will ever become invasive. Some methods being used or experimented with are tests using DNA flow cytometry, blood groups, oncogenes, cytogenetic abnormalities, and tumor proliferative activity. [7]

While it is true that most patients with superficial tumors don't have associated metastatic disease, it is also true that most patients with deep tumors do not have a history of superficial TCCs. [14, 15]

From 2.5% to 7% of superficial tumors invade a blood vessel. [7] If this could be known, it could be important for determining the risk of metastases of superficial tumors. While CIS itself may not access a blood vessel, the diagnosis is necessarily imperfect since all the tissue of the bladder is not tested so there may be spread to a blood vessel of a tumor thought to be CIS based on all the available evidence.

The WHO/ISUP article observed that CIS is often underdiagnosed and called moderate dysplasia. This is because some pathologists do not recognize that the diagnosis of CIS does not require the atypical cells extend over the entire thickness of the epithelium, the cells do not necessarily have a high nuclerar to cytoplasm ratio, the umbrella cell may still be intact, and a spectrum of atypia can exist with CIS. [1] All
these factors that can make CIS hard to diagnosis for a pathologist may also lead to difficulties with doing fluorescence spectroscopy. The disagreement in definition of what is CIS could also make it difficult to compare results to those of other experimenters.

6.20 Treatment for Bladder Cancer

Superficial tumors are usually treated with endoscopic destruction or resection. [7, 12, 14] Other methods of treatment include intravesical chemical agents or intravesical bacille Calmette Guerin (BCG). The latter is a biologic response modifier. [7, 14] However, others suggest no treatment is required for common superficial bladder tumors. [17] A large sized or multiple superficial tumors correlates with a greater risk of the tumor eventually becoming invasive. [12, 17]

If there is diffuse CIS, a cystectomy may be performed even though the cancer is superficial. [14] Other treatment options are irradiation or photodynamic therapy. In photodynamic therapy, a light sensitive chemical is introduced into the bladder and later exposed to intense laser light. [14]

For deep tumors of types T2, T3, and T4, partial or radical cystectomy is a common form of treatment. [14] Transurethral resection can be performed, but the incidence of recurrence and metastasis is high. Another treatment option is irradiation, but this fails 50% of the time on T3 patients. Yet another treatment option is chemotherapy. [14]

For metastatic tumors, the treatment is commonly systemic chemotherapy. [14]
6.21 Brunn's Nest

Brunn's nests are nests of urothelium or inbudding of the surface epithelium in the mucosal lamina propria. Brunn's nests may be normal variations in the morphology of the bladder. [5] If there is Brunn's nest involvement in bladder cancer, chemotherapy won't tend to reach it because chemotherapy doesn't reach far into the lamina propria. [7]

6.22 Carcinoma In Situ

Carcinoma in situ (CIS) or tumor in situ (TIS) is cancer confined to epithelium of the mucosa. [7] Although CIS usually occurs in flat urothelium, it can occasionally cover small inflammatory papillae, in which case it is still called CIS rather than papillary carcinoma. [7] In cases of CIS, the epithelial thickness varies, sometimes being thinner than normal because of less cohesiveness between tumor cells. [7]

CIS has three clinicopathologic types: a small focus of CIS immediately adjacent to a papillary tumor; CIS distant from papillary tumors; and CIS on all or nearly all of the urothelium, with or without associated papillary tumors. [7]

CIS can have pagetoid spread, or individual malignant cells within otherwise benign epithelium. Pagetoid spread does not change the clinical treated of CIS. [7]

An argument has been made to grade CIS as being grade 1 or 2, but most pathologists do not do this. There was also a proposal for a "bladder intraepithelial neoplasia" (BIN) system similar to the cervical and vulvar designations, but this was rejected by a panel of pathologists. [7]
6.23 Chemical Agents and Bladder Cancer

Various chemicals can greatly increase a patient's chance of getting bladder cancer. These chemicals can be encountered in some industrial settings and were previously used in the dye industry. For example, exposure to beta-naphthylamine makes bladder tumors occur 50 times more frequently. [11] Besides beta-naphthylamine, other carcinogenic compounds that lead to a greater risk of bladder cancer are 4-aminobiphenyl, 4-nitrobiphenyl, and 4,4-diaminobiphenyl. [5] A drug called cyclophosphamide, an immunosuppressant, causes hemorrhagic cystitis and atypia, which results in an increased risk of bladder cancer. [5, 7]

Male smokers have between two and four times as high an incidence of bladder cancer as male nonsmokers. [5] This indicates that the chemistry of cigarette smoke may influence the chance of getting bladder cancer.

Experiments indicate long-term in vitro samples of normal adult human urothelium, supported with stroma, can be maintained. This may prove valuable in learning more about chemical agents which lead to bladder cancer. Using animal models to investigate these chemicals may lead to incorrect conclusions because animals can differ in tumor formation compared to a human. [18]
6.24 Kidneys and Their Relationship to the Bladder

If the outflow of urine from one or both kidneys is blocked, such as by a tumor in the bladder blocking a ureter, a condition results called hydronephrosis. In hydronephrosis, the renal pelvis and calyces are dilated and the parenchyma atrophies. [11] This may be a complication of bladder cancer.

6.25 Mortality Statistics

In the U.S., there are around 35,000 new cases of bladder cancer per year and 10,000 die of the disease per year. [5, 9] For patients with squamous cell carcinoma, 70% are dead within a year. [5] For benign papilloma, the five-year survival is over 90% if the papilloma is resected or fulgurated; for malignant tumors not deeper than superficial layers of muscle, the five-year survival is 30-50%; and for tumors with deeper invasion, the five-year survival is 10-30%. [5, 11] 3% of cancer deaths in the U.S. are from bladder cancer. [5]

The cause of death with bladder cancer is either obstruction of the urine path leading to renal disease or it is dissemination of cancer to other organs of the body. [5]

6.26 Nonurothelial Tumors

95% of neoplasms in bladder have urothelial origin, but the other 5% are mostly mesenchymal tumors of myoblastic, fibroblastic, or endothelial origin. [5] Another source states that for bladder cancer, over 99% is carcinoma and less than 1% is sarcoma. [9]
Leiomyomas are the most common type of mesodermal tumor in the urinary bladder. These tumors are benign. [5] Sarcomas are malignant tumors from mesenchymal tissue or its derivatives; whereas carcinomas are malignant neoplasms from epithelial cells. [10] Sarcomas tend to form large masses with a soft, fleshy, gray-white gross appearance. [5]

One type of sarcoma seen in the bladder, rhabdomyosarcoma, comes in two varieties. The first is the "adult"-type, usually found only in adults over the age of forty, which has a histological appearance similar to striated muscle. The second is the embryonal type, usually found only during infancy or childhood, which produces large, polypoid projections of grapelike clusters of soft, fleshy tissue. [5]

6.26.1 Squamous Cell Carcinoma

Although only 5% of bladder cancers are true squamous cell carcinomas, other bladder cancers can have foci of squamous cell differentiation. [11] In squamous cell carcinoma, the tumor cells resemble stratified squamous epithelium. [10] Squamous cell tumors occur more frequently where schistosomal infections of the bladder are common, such as in Egypt. Whether the schistosomes are directly carcinogenic or whether the inflammatory response predisposes cancer is unclear. [5]

6.26.2 Adenocarcinoma

An adenocarcinoma occurs when neoplastic epithelial cells grow in gland patterns. [10] Malignant tumors that develop in urachal remnants and cysts are most
often adenocarcinoma. In the case of exstrophy, a condition discussed earlier in the section "Diverticulum," persistent chronic infections cause the mucosa to have an ulcerated surface of granulation tissue. The epithelium can develop metaplasia and proliferative cystitis. This increases the chance of the bladder developing carcinoma, especially adenocarcinoma. An exstrophy can be surgically corrected to prevent this. [5]

6.26.3 Other Transitional Epithelial Tissue Near the Bladder

Transitional epithelium lines tissue from the renal pelvis to the urethra, so epithelial tumors along this path will tend to have a similar morphologic pattern. [11] CIS may also spread to the prostate or down the urethra. CIS may also involve nonurothelial mucosa such as seminal vesicles, ejaculatory duct epithelium, urethral meats, and collecting ducts of the kidney. [7]

6.27 Recurrence Rates

All TCCs have a tendency to recur, in spite of excision. According to Robbins et al., between 60% to 80% of grade I papillary carcinomas recur and 80% to 90% of grade III lesions recur. [5] Whitmore reports that for superficial tumors, 30% to 90% recur. [14] Greene reports that for papillary tumors, 73% recurred. [12] The number of papillary tumors in the bladder affects the prognosis of recurrence, but is not a significant determinant of invasive disease. [7]
The chronology of the detection of the papillary tumor is important. For the first papillary tumor found in a patient and removed, there is a 45% risk of recurrence. However, if there has been a previous papillary tumor, the risk of recurrence of another papillary tumor is 84%. [7]

6.28 Factors Determining Risk for Recurrence and Progression

For TCC, the three most obvious factors which describe a tumor are sliding scales: noninvasive through invasive (stage); flat through papillary (gross morphology); and well-differentiated through highly anaplastic (grade). [5, 11] The gross morphology is not as clinically significant as the tumor stage, tumor grade, or the location of the tumor in the bladder, with the last being important because a tumor could block a ureter or urethra and cause urinary obstruction. [11]

A more extensive list of factors determining recurrence and progression of the tumor includes DNA analysis, tumor grade, depth of invasion, presence or absence of vascular invasion, number of tumors, size of tumors, status of blood group-antigen expression, S-phase fraction, chromosomal abnormalities, tumor proliferative activity, DNA ploidy, and associated mucosal abnormalities. [7] Various experimental tests are being evaluated and created to find more factors that can be used clinically.

6.29 Summary

This chapter looked at the anatomy of the bladder, which showed the importance of the ureters, the urethra, and the bladder itself for urine to exit the body. The layers in
the bladder wall were identified with particular attention paid to the urothelium, which is where the majority of bladder tumors originate. The anatomic differences between males and females show why males have a greater chance of developing difficulty voiding, because males have a prostate gland which can enlarge, whereas females have a greater chance of developing bacterial infection because they lack the antibacterial prostate fluid and also their urethra is much shorter. Diverticula provide a location where urine is held in stasis for extended periods, increasing the chance of bacterial growth and infection.

The reactive changes cells can undergo under various insults were listed because these changes can be mistaken for atypia seen in cancer cells. Bladder tumors were described as being papillary or flat, invasive or noninvasive. The stage of a tumor is given with a designation using the capital letter T. The grade is usually given on a scale that ranges from 1 to 3. Of the different types of bladder tumors, transitional cell carcinoma (TCC) is by far the most common. The most widely used tests for finding bladder cancer are an endoscopic examination and biopsy or a cytologic test, but many other tests exist or are being researched. For superficial tumors, treatment is often limited to resection or destruction of the tumor using an endoscope, but for deeper tumors, chemotherapy, radiation, and cystectomies are more commonly used. In spite of treatment, bladder cancer stubbornly tend to recur and may metastasize.
REFERENCES


CHAPTER 7

BLADDER IN VIVO CYSTOSCOPY

7.1 Material and Methods Specific For Cystoscopy Fluorescence

7.1.1 Experimental Setup

Tissue was accessed via an optical-fiber probe passed through the instrument channel of a standard rigid cystoscope. (Fig. 7.1) This equipment was discussed in the Chapter Three. A total of 19 patients had spectra taken in vivo through the cystoscope, but the equipment had additions made as the study progressed so that all 19 patients had fluorescence spectra taken at 370 nm excitation, only 14 patients had fluorescence spectra taken at 400 nm excitation, and only the last 5 patients had reflectance spectra taken.

Fluorescence spectra were collected at 370 nm excitation from 19 patients, but not all of these were usable. One patient was excluded because he had cuboidal dysplasia which does not fit the definition of transitional-cell-carcinoma (TCC) which is the subject of this thesis; another was excluded because the computer spectra files were corrupted; another patient was excluded because the notes for the day's experiment were lost; another was excluded because the tissue biopsy samples got lost without being diagnosed; and another was excluded because an obvious error in the background spectra caused it to be too large and subtracting it from the other spectra caused them to become negative.

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This left 14 patients at 370 nm calibration. Outliers were decided to be spectra more than three standard deviations from the mean at the peak for that diagnoses for all the included patients. Most of the spectra from the same patient were excluded this way which indicated a likely error in the calibration for that day. Therefore, two patients were taken out for having bad calibrations. Out of the remaining 12 patients at 370 nm excitation, one had neither normal benign mucosal tissue nor any papillary tumors. Thus, at 370 nm excitation, there were 11 patients with 24 papillary tumors and 33 benign mucosal tissue sites used for the mathematical analysis of this chapter. Of these 11 patients, 2 were female. In addition, but not included in the mathematical analysis, there were three dysplasia sites, one carcinoma-in-situ (CIS) site, one invasive cancer site, and two strong cystitis cystica sites. Only one of the dysplasia sites and the invasive site are in the 11 patients in the mathematical analysis, although the sites themselves are excluded as being neither benign mucosal tissue nor papillary tumor, with the rest from the patient with no benign mucosal tissue or papillary tumors at all.

Fluorescence spectra were collected at 400 nm excitation from 14 patients, but again not all of these were usable. One patient was again excluded because he had cuboidal; the patient whose biopsy samples were lost was excluded; and one patient was excluded for having a bad calibration. Out of the 11 remaining patients at 400 nm excitation, one patient had neither normal benign mucosal tissue nor any papillary tumors, this being the same patient that had this problem at 370 nm excitation. Thus, at 400 nm excitation, there were 10 patients with 22 papillary tumor and 23 benign mucosal tissue sites used for the mathematical analysis. Of these 10 patients, one was female.
addition, but not included in the mathematical analysis, there were three dysplasia, one CIS, and two strong cystitis cystica sites. The site at 370 nm excitation with invasive cancer was not measured at 400 nm excitation. Only one of the dysplasia sites was in the 10 patients in the mathematical analysis, although that site wasn't included in the mathematical analysis as not fitting the two categories.

Reflectance spectra were collected from the last five patients and all these were usable, but one was the patient who had neither normal benign mucosal tissue nor any papillary tumors. Thus, there were four patients with eight papillary tumor and six benign mucosal tissue sites used for the mathematical analysis. In addition, but not included in the mathematical analysis, there were two dysplasia, one CIS, and two strong cystitis cystica sites; these all came from the patient without any normal benign mucosal tissue or papillary tumors.

All the papillary tumors included in the mathematical analysis were classified by the pathologist as papillary Ta or papillary T1 TCC. Therefore, flat TCC and deeply invading TCC were not included in the mathematical analysis.

Spectra were collected from each site by placing the distal tip of the optical-fiber probe in light contact with the tissue surface. Following spectral acquisition, tissue was obtained from each site using cup biopsy forceps and submitted for routine histological examination.
7.1.2 Intensity-Calibration of Spectra

In order to compare fluorescence spectra acquired from different patients at different times, each spectrum was calibrated in a standardized manner. First, a measured background spectrum was subtracted to correct the spectral baseline to zero. Variations in laser power, collection efficiency, and system alignment were then eliminated by dividing each spectrum by the fluorescence intensity of a 3 mm-thick 485 nm colored glass long-pass filter measured at each excitation wavelength. Finally, the nonuniform spectral response of the detection system was corrected using the measured spectrum of a calibrated tungsten-halogen lamp.

A similar intensity-calibration was performed for the reflectance spectra, but the calibration standard came from spectra measured on hard-packed barium sulfate rather than on the colored glass filter.

7.1.3 Normalization of Spectra to Patient's Own Benign Mucosal Tissue Sites

Two methods were employed to analyze the fluorescence emission spectra. The first directly utilized the intensity-calibrated spectra. For the second, each spectrum was normalized wavelength-by-wavelength to the mean spectrum for the benign mucosal tissue sites of that patient. This reduced variation in the absolute intensity, particularly in cases of uncertain intensity calibration. In this method the patient’s benign mucosal tissue sites serve as an intensity calibration constant. This procedure is similar to that
used successfully in previous studies of the colon. [1, 2] In both intensity-calibrated and
normalized, the subsequent analysis employs the cross-validation method described
below. For the reflectance spectra, the normalization method was not employed.

7.1.4 Mean Spectra and Ratio of Mean Spectra

Primary differences in fluorescence intensity and spectral features were assessed
by examining the average ratio spectra, computed as the mean spectra of all the papillary
tumor sites of all the patients divided by the mean spectra of all the benign mucosal tissue
sites. The ratio spectrum provides a comparison of the average intensities at each
emission wavelength. If the mean papillary tumor spectrum and benign mucosal tissue
spectrum have the same spectral features, the ratio spectrum will be horizontal and flat; if
the spectra from the papillary tumor and benign mucosal tissue sites have the same
average intensity, the ratio spectrum will have an amplitude approximately equal to one.

7.1.5 Principal Component Analysis and Logistic Regression

Detailed analysis was carried out on spectra from individual tissue sites. A
multivariate statistical method was used to analyze the spectra and develop a diagnostic
algorithm to classify tissue sites as papillary tumor or benign mucosal tissue. The method
employed the emission spectra over the entire detected range (418-700 nm). This is
instead of the more common method of assuming a priori which wavelengths are
significant and then using only those for further analysis while removing the other
wavelengths, thereby potentially removing significant data. [3, 4, 5, 6, 7] Similar
multivariate methods have been used by others to analyze fluorescence, Raman, and infra-red (IR) spectra to classify sites or samples into various diagnoses or tissue types. [5, 6, 8, 9]

Principal component analysis (PCA) was used to characterize the features of the spectra. PCA separates the features, called principal components, from the independent noise with a purely mathematical technique by calculating the systematic covariance. Without user intervention, this mathematically resolves a spectral data set into a small number of principal components which accurately characterize the entire range of spectral variations (above the noise). Each individual fluorescence spectrum can thus be accurately represented as a linear combination of these principle components. The fit coefficients for each spectrum, called scores, can then be correlated with the findings of pathology and used to create a diagnostic algorithm. To avoid bias, the diagnostic algorithm should not be developed and tested on the same data set. [3, 4, 5, 6, 7, 10]

In our analysis the spectra were first mean-centered, the principal components for the entire data set were calculated via singular value decomposition using MATLAB, and scores were computed for each spectrum. [6] The principal components which were significant for classifying benign mucosal tissue and papillary tumor sites were found using multinomial logistical regression followed by likelihood-ratio tests in the Stata software. The principal components for which the likelihood-ratio test had a chi-squared value at or under the 0.05 significance level were treated as having diagnostic
significance. The scores corresponding to these selected principal components were correlated with the pathology diagnoses using multinomial logistic regression to create a diagnostic algorithm.

7.1.6 Cross-validation

Due to the small size of the overall data set, we employed a cross-validation scheme which optimizes the use of the data while still satisfying the need for each calibration set to be independent from the validation set. [4, 11, 12, 13] In our cross-validation scheme, the entire data set with one patient excluded was used as the calibration data set to develop a diagnostic algorithm, the results of which were then applied to diagnose the sites of the excluded patient. The process was continued with the excluded patient's data put back and another patient's data then excluded until the data from all the patients was cycled through. This meant each created diagnostic algorithm was tested on data to which it had been blinded. When the entire set of experimental data is one large set without blinding any data, that can explain variation. Using cross-validation, equivalent to the leave one out (LOO) method, where each algorithm is created with one set and tested on another set and then cycled through the entire set provides the predictive value of the model. [14, 15]

Since the intensity calibration signal and background signal were taken per patient rather than per emission spectrum, any errors in them affect not just one measured emission spectra, but all for that patient. Therefore, we excluded the data from one
patient rather than just one spectrum to prevent intra-patient bias, insuring that the
diagnostic algorithm is robust to a multi-patient sample. If it is assumed that each patient
has a similar number of sites, our method is comparable to k-fold cross-validation. [12]

7.2 Results

7.2.1 Patient Spectral Data

For the mathematical analysis, using 370 nm excitation, emission spectra were
collected from 24 papillary tumor and 33 benign mucosal tissue sites in eleven patients.
Using 400 nm excitation, emission spectra were collected from 22 papillary tumor and 23
benign mucosal tissue sites in ten patients. No photobleaching was detected during
acquisition at either excitation. Using the Xenon flashlamp, reflectance spectra were
collect from eight papillary tumor and six benign mucosal tissue sites in four patients.
Neither application of the optical-fiber probe nor exposure to laser light or the flashlamp
produced any observable tissue alterations on histologic examination.

For qualitative analysis with the mean spectra and ratio of mean spectra, another
patient was included for both 370 and 400 nm excitation fluorescence spectra and for the
reflectance spectra. At 370 nm excitation, there were then also three dysplasia, one CIS,
one invasive cancer, and two strong cystitis cystica sites. At 400 nm excitation, there
were then also three dysplasia, one CIS, and two strong cystitis cystica sites. With the
flashlamp and reflectance spectra, there were two dysplasia, one CIS; and two strong
cystitis cystica sites.
7.2.2 Mean Spectra and Ratio of Mean Spectra

The mean fluorescence spectra at 370 nm excitation from benign mucosal surface of the bladder, papillary tumors, dysplasia, CIS, cystitis cystica, and invasive cancer are shown in Fig. 7.2. Each emission spectrum is dominated by a peak at 460 nm. While CIS is an exception with the peak amplitude being larger than that for benign mucosal tissue, the rest seem to follow a pattern of decreasing amplitude with worsening disease. Invasive cancer is the worst disease and its peak is the lowest.

At 370 nm excitation, ratio spectra were formed from the mean spectra by dividing each diagnoses's average fluorescence intensity, other than benign mucosal tissue, at each emission wavelength by the corresponding average benign mucosal tissue fluorescence intensity. (Fig. 7.3) All the ratio spectra show a rising slope starting at 580-600 nm and climbing through 700 nm. This is the red part of the light spectrum so corresponds to a rise in the red. The ratio of papillary tumor to benign mucosal tissue has the weakest red rise compared to the other disease types.

The mean fluorescence spectra at 400 nm excitation from benign mucosal surface of the bladder, papillary tumors, dysplasia, and cystitis cystica are shown in Figure 7.4. Again, each emission spectrum is dominated by a peak at 460 nm. As before, increasing severity of disease seems to decrease the overall amplitude of the emission peak. Ratio spectra were formed. (Fig. 7.5) Again, all the ratio spectra show a rising slope starting at about 580 nm and climbing through 700 nm, although the ratio of CIS to benign mucosal tissue has a small red rise. The ratio of papillary tumor to benign mucosal tissue and the ratio of dysplasia to benign mucosal tissue having the strongest red rises.
The mean reflectance spectra, presented in absorption units by taking the negative log base ten of the measured reflectance after background subtraction and calibrating with the spectra taken of barium sulfate, are shown in Figure 7.6. [23] For all the diagnoses, three distinct peaks are noticeable at 420, 530, and 580 nm. These absorption peaks correspond to the peaks of hemoglobin as reported in the literature. [16] The more diseased the tissue, the greater the absorption seems to be so that the overall spectral curves rise as the disease is progressively worse.

Ratio spectra were formed for the reflectance spectra. (Fig. 7.7) The ratio of papillary tumor to benign mucosal tissue shows an absorption peak at 420 nm likely from the Soret band of hemoglobin, indicating the papillary tumors have more hemoglobin than the benign mucosal tissue. The ratio of CIS to benign mucosal tissue shows dips at 420 and 580 nm, which could indicate less hemoglobin than for benign mucosal tissue. However, the CIS spectra come from only a single site so generalizations have to be treated with caution. The ratio of dysplasia to benign mucosal tissue is near one and relatively flat compared to the other diagnoses, which could indicate difficulty in diagnosing dysplasia from benign mucosal tissue using reflectance spectra. The ratio of cystitis cystica to benign mucosal tissue has large peaks at 420, 530, and 580 nm likely indicating more hemoglobin in the cystitis cystica tissue than in the normal benign mucosal tissue. Other than cystitis cystica to benign mucosal tissue ratio which has larger changes in its spectra than the other categories, the others look relatively flat in the red portion of the spectra.
The mean fluorescence spectra from the benign mucosal surface of the bladder and papillary tumor from all patients used in the mathematical analysis, along with one standard deviation above and below that to indicate the error, are shown in Figure 7.8 (370 nm excitation) and Figure 7.9 (400 nm excitation). Each spectrum is dominated by a strong emission band that peaks near 460 nm, with weak fluorescence at wavelengths greater than 600 nm. The overall fluorescence intensity of benign mucosa was greater than that of papillary tumor at both excitation wavelengths. Excitation at 400 nm produced more evidence of fluorescence in the red region of the spectrum than did 370 nm excitation. Looking at the standard deviation lines, there is a gap between the emission peak curve for the benign mucosal tissue minus one standard deviation and the emission peak curve plus one standard deviation at both excitations. This indicates separation between the two groups.

Ratio spectra were formed from the mean spectra by dividing the papillary tumor fluorescence intensity at each emission wavelength by the corresponding benign mucosal tissue fluorescence intensity. (Fig. 7.10) The ratio spectra at both excitation wavelengths are characterized by two distinct regions. The flat region around 500 nm is similar for both benign mucosal tissue and papillary tumor. This region has a constant value less than one, indicating that fluorescence from papillary tumors is less intense than from benign mucosa. Second, the rising ratio spectrum beyond 600 nm suggests the presence of an additional fluorophore, predominant in papillary tumors, that gives rise to added
fluorescence in this region. This increased fluorescence was seen occasionally in individual papillary tumor spectra, sometimes producing discernible peaks at 630 and 680 nm.

The mean reflectance spectra from the benign mucosal surface of the bladder and papillary tumor from all patients used in the mathematical analysis, along with one standard deviation above and below that to indicate the error, are shown in Figure 7.11. It shows absorption peaks at 420, 530, and 580 nm as would be expected from hemoglobin. It also shows that the average papillary tumor spectrum minus one standard deviation is higher than the average benign mucosal tissue spectrum plus one standard deviation. This indicates the two diagnosed tissue types do tend to separate and is promising for a diagnostic algorithm based solely on reflectance rather than fluorescence.

7.2.3 Principal Component Analysis and Logistic Regression

For the spectra at each excitation wavelength, PCA and logistic regression were employed, excluding the data from one patient at a time, to obtain a diagnostic algorithm which was then applied to the data of the excluded patient, as described earlier.

The first four principal components were sufficient to provide good fits, while the first nine were sufficient to provide fits within the noise. If using and testing only the first four led to more than one component being diagnostically significant and then if using and testing the first nine did not include those same diagnostically significant principal components, only the first four were used in the subsequent analysis, otherwise the first nine were used.

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Two principal components were found significant for the 400 nm excitation intensity-calibrated data, for the 400 nm excitation normalized data, and for the 370 nm excitation intensity-calibrated data (1 and 3, 1 and 2, and 1 and 2, respectively); four principal components were found significant for the 370 nm excitation normalized data (1, 3, 5 and 8); and one principal component was found significant for the reflectance spectra (1). These were then used to produce the diagnostic algorithm.

The principal component spectra and decision lines were found to change only slightly when data from different patients were excluded. Thus, to illustrate the results graphically, we consider the principal components from the entire spectral data set (i.e. all patients included). However, using all the patients does not blind anything when creating the diagnostic algorithm so the sensitivity and specificity will likely be higher, but this has little meaning in evaluating the predictive value.

7.2.4 Intensity-Calibrated Fluorescence Data

Figure 7.12 shows the first three principal components obtained from the intensity-calibrated data for 370 nm excitation. The first principal component has the basic peak shape that can be seen in the average curves of Figure 7.8. The second principal component has a large drop from where the spectra starts at 418 nm rising up until about 480 nm, which could be related to the 420 nm Soret band absorption of hemoglobin. The third principal component looks like an inverted hemoglobin
absorption curve with dips at 420, 530, and 580 nm, yet it was not found to have
diagnostic significance. These shapes were found solely mathematically and did not have
meaning for a particular fluorophore or absorber assigned to them \textit{a priori}.

Figure 7.13 shows a binary decision plot with the scores for the diagnostically
significant principal components of one and two for 370 nm excitation. The points are
strongly grouped. The graph also has a decision line with those points in the upper right
called benign mucosal tissue and those to the lower left called papillary tumor. The
pathologist's diagnoses for benign mucosal tissue is marked with a solid diamond and for
papillary tumors with a hollow square. This method finds 22 of the 24 papillary tumors
and 28 of the 33 benign mucosal tissue sites for a sensitivity of 92\% and a specificity of
85\%. However, this was not done with the creation of the algorithm blinded to the data
used in it so does not give the predictive value of the model. That comes from the cross-
validation which does test data not used to create the algorithm itself.

Figure 7.14 shows the first three principal components obtained from the
intensity-calibrated data for 400 nm excitation. The first principal component has the
basic peak shape that can be seen in the average curves of Figure 7.9. The second
principal component mainly exhibits spectral behavior in the range 418-440 nm, which
may be related to the Soret band absorption of hemoglobin, yet it did not prove to have
sufficient diagnostic significance to be included in the calibration model. The third
principal component primarily exhibits spectral structure at wavelengths of 590 nm and
longer, which corresponds to the rise in the red portion of the spectrum seen in the ratio
spectra of Figure 7.10, and it was found to have diagnostic significance.
Figure 7.15 shows a binary decision plot with the scores for the diagnostically significant principal components of one and three for 400 nm excitation. It also has a decision line with those points in the upper right called benign mucosal tissue and those to the lower left called papillary tumor. The pathologist's diagnoses for benign mucosal tissue is marked with a solid diamond and for papillary tumors with a hollow square. This method finds all 22 papillary tumors and all 23 benign mucosal tissue sites for 100% sensitivity and 100% specificity. However, this was not done with the creation of the algorithm blinded to the data used in it so does not give the predictive value of the model.

Applying the leave-one-patient-out cross-validation scheme to the 400 nm excitation data set gave the following results: Of 23 benign mucosal tissue sites, one was misdiagnosed, and of 22 papillary tumor sites, four were misdiagnosed, resulting in 82% sensitivity and 96% specificity. Note this was done in blinded fashion and is considerably worse than the 100% sensitivity and 100% specificity when the entire set was used at one time in a non-blinded fashion.

A similar procedure cross-validation was carried out for the 370 nm excitation spectra, which contained spectra from eleven patients. Of the 33 benign mucosal tissue sites, three were misdiagnosed, and of the 24 papillary tumor sites, three were misdiagnosed, resulting in 88% sensitivity and 91% specificity. In this case, sensitivity got worse than the 92% of the entire non-blinded set but the specificity got better than the 85% of the entire non-blinded set. Only the blinded results give the predictive value of the model.
7.2.5 Normalized Fluorescence Data

The above analysis was similarly applied to the normalized data. Figure 7.16 shows the average normalized spectra for benign mucosal tissue and for papillary tumors, along with plus and minus one standard deviation, for 400 nm excitation. As described in the Methods section, the normalization divides each spectrum by the average of the benign mucosal tissue spectrum for that same patient. Since the normalization is to the benign mucosal sites, the average of all the benign mucosal sites is a flat horizontal line. The average normalized papillary tumors curve looks similar to the non-normalized ratio spectra of the average papillary tumors for all the patients divided by benign mucosa for all the patients. It does have some differences because the normalization procedure is on a patient-by-patient basis and not done on all the patients together.

Figure 7.17 shows the first three principal components obtained from the normalized data for 400 nm excitation. The first principal component is mainly a flat constant signal, which is reasonable, since the normalization procedure transforms the basic peak shape into a flat line, from which individual spectra can vary either positively or negatively. The second principal component primarily pulls out wavelengths from 590 nm and longer; it is similar to the third principal component for intensity-calibrated spectra but with a change in sign which simply changes the sign of the corresponding score In this case only the first and second principal components had diagnostic significance at the 0.05 level.

Figure 7.18 displays the scores of the two diagnostically significant principal components, 1 and 2, on a binary decision plot, along with the calculated decision line
As with the intensity-calibrated binary decision plot of Figures 7.13 and 7.15, the papillary tumor and benign mucosal tissue sites are clearly grouped, with the decision line clearly separating them. However, as can be seen by comparing with Figure 7.13, normalization tightens the groupings. For the entire data set, all 22 papillary tumors and all 23 benign mucosal tissue sites were correctly found for 100% sensitivity and 100% specificity, but this is not the diagnostic potential because the data was not blinded.

When run in blinded fashion with cross-validation, of the 23 benign mucosal tissue sites, one was misdiagnosed, and of the 22 papillary tumor sites, two were misdiagnosed, resulting in 91% sensitivity and 96% specificity. Once again, blinding the algorithm to the data it is used on results in a worse sensitivity and specificity, but with meaning for the predictive value of the model.

The 370 nm excitation normalized excitation spectra contained data from eleven patients. For this data set, four principal components (1, 3, 5 and 8) were found to be significant, all of which were used, with good results. A simple binary decision plot could not be made because it would require four dimensions. Testing all the data with the four dimensional decision line found 23 of the 24 papillary tumor and 32 of the 33 benign mucosal tissue sites for a 96% sensitivity and 97% specificity. With the cross-validation analysis, three of the 24 papillary tumor sites and one of the 33 benign mucosal tissue sites were misdiagnosed, resulting in 88% sensitivity and 97% specificity, which is again worse than using the full set in an non-blinded fashion.
7.2.6 Reflectance Data

The reflectance spectra analyzed mathematically included four patients. For this data set, only the first principal component was found to be significant. (Fig. 7.19) It provides little more than a DC amplitude. With the cross-validation analysis, six of the eight papillary tumors and all six of the benign mucosal tissue sites were diagnosed which gives 75% sensitivity and 100% specificity.

7.3 Discussion of In Vivo Bladder Fluorescence

The result of a seemingly increasing severity of disease for flat lesions including dysplasia, CIS, and invasive cancer leading to steadily decreasing overall amplitude of the emission peak at both excitations is an intriguing result. (Fig. 7.2 and 7.4) Also intriguing is the red rise seen in the ratio graphs for all the disease diagnoses at both excitations. (Fig. 7.3 and 7.5) However, the various flat lesions were only observed in one or two patients and only one, two, or three times each prevented meaningful mathematical analysis. The patient selection criteria involved seeing patients having papillary tumors removed. The lack of other disease types in these patients might indicate a low risk of a papillary tumor ever progressing into an invasive cancer, as is noted and discussed in depth in Chapter Six.

The absorption also seemed to increase as the severity of the disease increased. (Fig. 7.6) This result could indicate that all of or some of the change in amplitude of the fluorescence spectra came not from changes in the fluorophores, but rather from changes in the absorption of the tissue. However, the ratio graphs for the reflectance does not
indicate a significant change in the red portion of the spectra as seen in the fluorescence spectral graphs, indicating there probably truly is a second fluorophore with an emission peak in the red portion of the spectra distinctive from changes in absorption properties. (Fig. 7.7)

The mathematical part of study evaluated the potential of fluorescence spectroscopy to distinguish papillary TCC from benign bladder mucosa during cystoscopy. Since a cross-validation method was used which left out one patient at a time, that meant there was a slightly different diagnostic algorithm for each patient. The other diagnoses of dysplasia, CIS, invasive cancer, and cystitis cystica were not tested with the algorithm developed for benign mucosal tissue and papillary tumors because there was actually a separate algorithm for every patient.

The results for benign mucosal tissue and papillary tumors indicate that significant differences exist, particularly in the overall amplitude and in the red portion of the spectra. This can be seen in Figure 7.10, which show the average ratio of papillary tumor to benign mucosal tissue. The curves are fairly flat from 418 to 580 nm, reduced from a value of 1.0 to about 0.4 at 370 nm excitation and about 0.15 at 400 nm excitation. At both excitation wavelengths the curves climb upward at the red portion of the spectra (580 to 700 nm), although this behavior is more pronounced for 400 nm excitation. The same important features appear in Figure 7.14 for 400 nm excitation, where principal components 1 and 3 proved to be diagnostically significant. Principal component 1 has the shape of the average spectra with the peak at 460 nm, which would correlate to changes in overall amplitude. The amplitude of principal component 3 drops beyond 580
nm, the red portion of the spectra. Since principal components 1 and 3 have these shapes and are fitted to the calibration set, using them should provide the same basic information seen in the ratio graph. After normalizing to the patient’s own benign mucosal tissue sites, the diagnostically significant principal components, 1 and 2, can be seen in Figure 7.17 for 400 nm excitation. The normalization process flattens the spectra, which results in principal component 1 now appearing as a flat constant signal. Principal component 2 has the same basic red rise as is seen in the ratio graph. The principal component analysis (PCA) algorithm found the importance of the overall amplitude and the red rise solely by mathematical analysis, not through additional input by the user. This suggests PCA is a valid and useful tool for determining the spectral features which are important in making diagnostic decisions.

Previous in vivo studies of the fluorescence spectra of normal and dysplastic colonic mucosa conducted in our laboratory provided similar results in regard to the importance of the overall amplitude compared to the change in the spectra in the red spectral region. [1, 2] In those studies, an identical instrument was used to collect spectra at colonoscopy using only 370 nm excitation. Similar normalized parameters were used, in a blinded fashion, to identify dysplasia with a sensitivity, specificity, and positive predictive value of 90%, 95%, and 90%, respectively. [2] Thus, this technique may be applicable to detection of cellular atypia in mucosa or epithelium of other organs.

The utility of two distinct and independent regions of the fluorescence spectrum in both this and previous studies suggests that there are at least two important fluorescent structures, represented by at least two independent fluorophores, that are responsible for
the observed spectral differences. Autofluorescence microscopy studies have identified these structures in the colon as collagen, primarily responsible for fluorescence in the blue region, and one or more fluorophores in the cytoplasm of dysplastic cells, contributing blue fluorescence and additional fluorescence in the red. [17, 18] Although not identified conclusively, the fluorescence at wavelengths greater than 600 nm was consistent with that of endogenous porphyrins, specifically protoporphyrin IX, which have been shown to be present preferentially in papillary tumor cells as was reported by Yuanlong et al. [19] Both Harris et al. and Yuanlong et al. found this porphyrin fluorescence in tumors in other organs too. [19, 20]

Richards-Kortum attributed emission fluorescence in the red part of the spectrum to porphyrins in bacteria, referring to squamous cell carcinoma rather than transitional cell carcinoma. [21] The pathologist in our study did not report any obviously visible bacteria in the stained slides. Whether the cause of what seems to be porphyrin fluorescence in the papillary tumors in this study comes from bacteria growing on the papillary tumors and not on the benign mucosal tissue or rather from biological changes within the papillary tumor itself is still an open question.

Morphological changes also contribute to the emission fluorescence spectral response of benign bladder tissue and papillary tumors as suggested by Koenig et al. [22] A thickened epithelium in papillary tumors causes the lamina propria to be further from the tissue surface. This results in less excitation light reaching the lamina propria to cause fluorescence and less fluorescence emission light getting back to the tissue surface.
Therefore, for the fluorescence emission spectra collected at the tissue surface, a smaller portion will be contributed from fluorescence in the lamina propria and more from the epithelium itself compared to the spectra collected from benign tissue.

Examination of the fluorescence spectra of Figure 7.9, with 400 nm excitation, suggests heme absorption within both benign mucosa and papillary tumors, as evident by local valleys in the spectra at 420 and 580 nm. [23] The reflectance spectra in absorbance units shows absorption peaks at 420, 530, and 580 nm also indicating likely presence of heme in the tissue. (Fig. 7.6) [23] Since the contact probe displaces the contents filling the bladder, effectively removing suspended blood, this absorption likely takes place within the tissue itself rather than at its surface, and may be due to hemoglobin breakdown products or another heme protein rather than hemoglobin itself.

As discussed in the Chapter One of this thesis, Auden found excitation peaks for mammalian cells at 360 nm and 400 nm, which closely match the 370 and 400 nm excitations used in this study. [24] The results here indicate 400 nm was the preferable excitation wavelength and this seemed to be from 400 nm better exciting porphyrin molecules giving a stronger red rise in the emission spectra of diseased tissue.

Baert et al. used 405 nm excitation, very close to the 400 nm excitation used in this study, to study bladder fluorescence, but that was after using Photofrin dye, a hematoporphyrin derivative, which artificially adds porphyrin fluorescence. [25] Their choice of excitation wavelength was designed to excite the porphyrin dye. In the blue part of the emission spectrum, which they claim is simply autofluorescence and not influenced by the dye, they found at least a ten times decrease in the 460 nm emission
peak of papillary tumors compared to normal benign mucosal tissue. This study found 7.0 times decrease in this 460 nm emission peak at 400 nm excitation which although somewhat smaller is still of the same order as their results and is a dramatic decrease.

D'Hallewin et al. successfully used fluorescence spectroscopy to detect bladder TCC and CIS without the use of exogenous dyes at excitations of 355 and 365 nm. [26, 27, 28, 29] In their study at 365 nm, which is very close to our 370 nm excitation, they found the 460 nm emission peak of papillary tumors to be 3.2 times lower than that of normal benign mucosal tissue. [28] This compares well with the 2.6 times lowering of the peak found in our study at 370 nm excitation. However, D'Hallewin et al. indicated that other than the amplitude difference, their spectral shapes for benign mucosal tissue and papillary tumor were virtually superimposable while our study found a mild red rise for the papillary tumors even at 370 nm excitation. [28]

D'Hallewin et al. reported 100% sensitivity and 100% specificity at 365 nm excitation, but this used the entire data set without blinded data. [28] For our study at 370 nm excitation a 92% sensitivity and 85% specificity was found when using the entire data set, but using cross-validation resulted in a 88% sensitivity and 91% specificity.

Perhaps a better way to compare is to our 400 nm non-normalized excitation results where using the entire data set resulted in 100% sensitivity and 100% specificity, while using cross-validation resulted in 82% sensitivity and 96% specificity. This indicates that there is not too much meaning comparing the results of a cross-validation study to find the predictive value of a model to simply processing the entire data set together without any blinding.
Koenig et al. used 337 nm excitation. [22, 30] With this, they reported 97% sensitivity and 98% specificity, but that was using the entire data set together without any blinding. This should be compared to our 400 nm excitation results of 100% sensitivity and 100% specificity from using the entire set together rather than to our cross-validation results of 82% sensitivity and 96% specificity. [30] This leaves the question of whether 337 or 400 nm excitation is preferable as not yet answered. We did find 370 nm excitation not to be as useful as 400 nm and these results reported by Koenig could indicate 337 nm excitation might be preferable to 370 nm excitation too.

For our cross-validation results, the normalization procedure did improve the results compared to the non-normalized data. With 370 nm excitation, the non-normalized results were 88% sensitivity and 91% specificity while the normalized results were 88% sensitivity and an improved 97% specificity. With 400 nm excitation, the non-normalized results were 82% sensitivity and 96% specificity while the normalized results were a considerably improved 91% sensitivity and still 96% specificity.

The present study demonstrates the feasibility of fluorescence spectroscopy to distinguish between benign mucosa and papillary tumor during cystoscopy, and forms the groundwork for detecting less visually apparent lesions including epithelial dysplasia and CIS. This opens the possibility for developing an endoscopic system that produces diagnostic tissue maps based on the results of tissue spectroscopy as an adjunct to histologic analysis and other clinical parameters. Such technology could form the basis of a simple and effective method of surveillance of the entire mucosal surface of the bladder and may help direct clinical management.
REFERENCES


Figure 7.1: Schematic diagram of the in situ laser spectrofluorometer, illustrating the distal tip of the optical-fiber spectral probe in the inset.
Figure 7.2: Mean fluorescence spectra at 370 nm excitation for all the various tissue diagnoses.
Figure 7.3: Ratio fluorescence spectra at 370 nm excitation computed by dividing the mean of all diseased tissue spectra by the mean of all normal benign mucosal tissue spectra.
Figure 7.4: Mean fluorescence spectra at 400 nm excitation for all the various tissue diagnoses.
Figure 7.5: Ratio fluorescence spectra at 400 nm excitation computed by dividing the mean of all diseased tissue spectra by the mean of all normal benign mucosal tissue spectra.
Figure 7.6: Mean reflectance spectra for all the various tissue diagnoses.
Figure 7.7: Ratio reflectance spectra computed by dividing the mean of all diseased tissue spectra by the mean of all normal benign mucosal tissue spectra.
370 nm excitation

Figure 7.8: Mean fluorescence spectra and plus and minus one standard deviation for benign mucosal tissue and papillary tumors, at 370 nm excitation.
Figure 7.9: Mean fluorescence spectra and plus and minus one standard deviation for benign mucosal tissue and papillary tumors, at 400 nm excitation.
Figure 7.10: Ratio fluorescence spectra computed by dividing the mean of all papillary tumor spectra by the mean of all normal benign mucosal tissue spectra, excited using 370 nm and 400 nm laser light.
Figure 7.11: Mean reflectance spectra and plus and minus one standard
deviation for benign mucosal tissue and papillary tumors.
370 nm excitation

Figure 7.12: First three principal components calculated for 370 nm excitation spectra without normalization to patient's own normal benign mucosal tissue spectra.
370 nm excitation

Figure 7.13: Binary scatter plot of scores one and three for 370 nm excitation spectra without normalization to patient's own normal benign mucosal tissue spectra.
Figure 7.14: First three principal components calculated for 400 nm excitation spectra without normalization to patient's own normal benign mucosal tissue spectra.
Figure 7.15: Binary scatter plot of scores one and three for 400 nm excitation spectra without normalization to patient's own normal benign mucosal tissue spectra.
Figure 7.16: Mean fluorescence spectra and plus and minus one standard deviation for benign mucosal tissue and papillary tumors with normalization to patient's own normal benign mucosal tissue spectra, at 400 nm excitation.
Figure 7.17: First three principal components calculated for 400 nm excitation spectra with normalization to patient's own normal benign mucosal tissue.
Figure 7.18: Binary scatter plot of scores one and two for using 400 nm excitation spectra with normalization to patient's own normal benign mucosal tissue.
Figure 7.19: First three principal components calculated for the reflectance spectra.
CHAPTER 8

FLUORESCENCE SPECTROSCOPY OF THE IN VITRO BLADDER

8.1 Materials and Methods Specific for Fluorescence Spectroscopy After Cystectomy

8.1.1 Experimental Setup

Autofluorescence and reflectance spectra were taken at specific sites on the urinary bladder wall within thirty minutes of resection of the urinary bladder due to cancer. For each patient, the physician took a few moments during the operation and selected sites that look grossly normal or diseased to his naked eye in the resected bladder. Typically, 6 to 8 sites were chosen and measured with the spectrometer in each bladder. This chapter deals with the autofluorescence spectra and the next chapter deals with the reflectance spectra. These spectra were taken with an optical-fiber probe that was narrow enough to have been passed through the instrument channel of a standard cystoscope and was the same set of probes used in the in vivo study. This equipment was discussed in Chapter Three and is identical to that used in Chapter Seven, from now on referred to as the in vivo study. A total of 35 patients had fluorescence spectra taken at
both 370 and 400 nm excitation. Soon after the study began, a Xenon flashlamp was added to the equipment so while the first six patients did not have white light reflectance measurements taken, reflectance spectra were taken for the other 29 patients.

At each site for which spectra were taken in the resected bladder, biopsies were taken. Either all the biopsies were put into Holland’s fixative or else two biopsies were instead frozen in liquid nitrogen for the microscopy study and the rest put into fixative. The fixed tissue was cut, put on microscope slides, and stained with hematoxylin and eosin (H&E). The frozen tissue was cut and alternate sections were either imaged on the fluorescence microscope or otherwise H&E stained. The H&E stained microscope slides were diagnosed by a pathologist. These detailed diagnoses were then simplified into five categories. The categories were benign mucosal tissue, dysplastic tissue, carcinoma-in-situ (CIS), invasive cancer, or papillary tumors. The computer data files containing the spectra were transferred from the personal computer on the instrument cart brought into the O.R. to a Unix workstation for further processing. Analysis of the measured fluorescence and reflectance spectra determined how well they could be used to diagnose the tissue into the appropriate categories as had been determined by the pathologist.

The results of the fluorescence microscopy performed on the frozen biopsies is described in Chapter Ten.

8.1.2 Intensity-Calibration of Fluorescence Spectra

As with the spectra in Chapter Seven with the bladder in vivo study, in order to compare fluorescence spectra acquired from different patients at different times, each
spectrum was calibrated in a standardized manner. First, a measured background spectrum was subtracted to correct the spectral baseline to zero. Variations in laser power, collection efficiency, and system alignment were then eliminated by dividing each spectrum by the fluorescence intensity of a 3 mm-thick 485 nm colored glass long-pass filter measured at each excitation wavelength. Finally, the nonuniform spectral response of the detection system was corrected using the measured spectrum of a calibrated tungsten-halogen lamp.

For the colon study in Chapter Five and bladder in vivo study in Chapter Seven, a second method of calibration was done where each spectrum was normalized wavelength-by-wavelength to the mean spectrum for the benign mucosal tissue sites of that same patient. This normalization procedure reduced variation in the absolute intensity since the patient's benign mucosal tissue sites serve as an intensity calibration constant. For this normalization to be used meaningfully for diagnostic purposes, there must be a priori knowledge of which non-diseased vs. diseased tissue, perhaps based on gross appearance of the tissue. If the pathologist's diagnoses is required to know which is the non-diseased tissue for this normalization procedure, then to say a diagnostic procedure has been developed using that normalization to match the pathologist's diagnoses has is not valid since the pathologist's diagnoses were used to create the algorithm. For the colon study, the polyps were grossly different in appearance compared to the surface of the colon without polyps, so this normalization procedure was valid there. Similarly for the bladder in vivo cystoscopy study, the papillary tumors had a grossly different appearance from the flat bladder wall, so this normalization procedure was valid there. For this chapter
dealing with the bladder *in vitro* study, flat disease of dysplasia and CIS were common. Grossly, these could not be determined by the naked eye. Even the experienced physician who attempted to find these in the tissue based on gross appearance often instead found inflamed or hemorrhagic tissue that had neither dysplastic tissue nor CIS while sometimes what he guessed was healthy benign tissue had the diseases of dysplasia or CIS. Therefore, this normalization procedure is not used in this chapter.

8.1.3 Mean Spectra and Ratio of Mean Spectra

As with the colon study in Chapter Five and bladder *in vivo* study in Chapter Seven, primary differences in fluorescence intensity and spectral features were assessed by examining the average ratio spectra, computed as the mean spectra of all the diseased sites of all the patients divided by the mean spectra of all the benign mucosal tissue sites. While the colon study had only three categories—normal colon tissue, hyperplastic polyps, and adenomatous polyps—and while the bladder in vivo study had only two categories—benign mucosal tissue and papillary tumors—there were five categories in this bladder *in vitro* study—benign mucosal tissue, dysplastic tissue, CIS, invasive cancer, and papillary tumors. If the spectra from the diseased tissue and the benign mucosal tissue have the same average intensity, the ratio spectrum will have an amplitude approximately equal to one. Otherwise, the ratio spectra highlight differences in spectral shape.
8.1.4 Benign Mucosal Tissue Subdivided Into More Categories

The pathologist frequently observed hemorrhage, edema, inflammation, and denuding in the samples on the H&E slides. For better understanding of the effect these complications in the tissue had, the benign mucosal tissue slides were brought back to the pathologist. Each category of hemorrhage, edema, inflammation, and denuding was rated on a scale of 0, 1, 2, or 3.

For hemorrhage, category zero meant none, one meant focal mild, three meant marked diffuse, and two meant between focal mild and diffuse. For edema, category zero meant none, one meant focal mild, two meant between 10% and 75%, and three meant greater than 75%. For inflammation, category zero meant none, category one meant focal mild with cluster of a few cells, three meant marked diffuse, and two meant more than a few clusters but less than marked diffuse. For denuding, category zero meant none, one meant less than 10%, two meant between 10% and 90%, and three meant greater than 90%.

8.1.5 Principal Component Analysis and Logistic Regression

As in the colon study in Chapter Five and the bladder \textit{in vivo} study in Chapter Seven, detailed analysis was carried out on spectra from individual tissue sites with a multivariate statistical method used to analyze the spectra and develop a diagnostic algorithm. The emission spectra over the entire detected range (418-700 nm) was used instead of assuming \textit{a priori} which wavelengths were significant. This method is discussed in more depth in Chapter Four.
Principal component analysis (PCA) separates the features, called principal components, from the independent noise with a purely mathematical technique by calculating the systematic covariance. The fit coefficients for each spectrum, called scores, can then be correlated with the findings of pathology and used to create a diagnostic algorithm. To avoid bias, the diagnostic algorithm should not be developed and tested on the same data set, as discussed in Chapter Four.

In our analysis the spectra were first mean-centered, the principal components for the entire data set were calculated via singular value decomposition, and scores were computed for each spectrum. The principal components which were significant were found using multinomial logistical regression (LR) followed by likelihood-ratio tests. The principal components for which the likelihood-ratio test had a chi-squared value at or under the 0.05 significance level were treated as having diagnostic significance. The scores corresponding to these selected principal components were correlated with the pathology diagnoses using multinomial logistic regression to create a diagnostic algorithm. More information on this technique is available in Chapter Four.

8.1.6 Cross-validation

The in vitro cystectomy data set is considerably larger than the in vivo cystoscopy data set, but counterbalancing that is the cystectomy data set was broken up into five diagnostic categories rather than simply benign mucosal tissue and papillary tumors. To compensate for set sizes of different relative sizes, the diagnoses were separated into non-diseased and diseased categories with different types of disease examined separately. For
example, one test was to have the non-diseased category as benign mucosal tissue and the
diseased category as both the diseases of dysplasia and CIS, leaving invasive cancer and
papillary tumors out of the analysis. This let sensitivity and specificity be traded off one
for the other.

Although the data sets were small, it was decided to first used the entire set of the
different disease types. Only if the sensitivity and specificity were both over 80% was
the analysis continued with the cross-validation leave-one-patient-out method.

8.2 Results of Fluorescence Spectroscopy at Cystectomy

8.2.1 Number of Patients and Sites For Each Diagnosis Category

For 370 nm excitation, there were 35 patients with spectra taken, but only 30 of
these were used in the analysis. The reasons the five patients were excluded was either
they had bad backgrounds (subtraction of these backgrounds caused the fluorescence
spectra to go strongly negative) and bad calibrations (as determined by more than three
standard deviations from the mean for the benign mucosal tissue category at the 460 nm
emission peak). Three of the 30 patients were female. For 400 nm excitation, there were
35 patients with spectra taken, but only 30 of these were used in the analysis and these
were not all the same 30 patients that were used at 370 nm excitation. Two of the 30
patients used at 400 nm excitation were female. The bad backgrounds or bad calibrations
sometimes occurred at one excitation wavelength but not the other.

Closer examination of the individual spectra with the diagnosis of invasive cancer
revealed an interesting phenomena also described earlier in Chapter Five. Some spectra
had very large emission peaks at 630 and 680 nm while most of the others did not.

(Fig. 8.1 and 8.2) With 400 nm excitation, one spectrum had a 630 nm peak taller than the primary 460 nm peak. (Fig. 8.2) Since PCA is a variance based technique, these large peaks were so strongly targeted that the results were poor. Therefore, the one spectrum at 370 nm excitation that climbed over the mean times three times the standard deviation at both 630 and 680 nm was excluded from further analysis and same for the one spectrum at 400 nm excitation that also climbed over the mean times three times the standard deviation at both 630 and 680 nm.

For the 30 patients at 370 nm excitation used in further analysis, there were 90 sites of benign mucosal tissue from 27 patients, which means three patients had no benign mucosal tissue measured at all. There were also 21 sites of dysplastic tissue from 12 patients, 26 sites of CIS from 15 patients, 33 sites of invasive cancer from 13 patients, and 4 sites of papillary tumor from one patient.

For the 30 patients at 400 nm excitation used in further analysis, there were 84 sites of benign mucosal tissue from 27 patients, 20 sites of dysplastic tissue from 11 patients, 25 sites of CIS from 13 patients, 38 sites of invasive cancer from 14 patients, and 6 sites of papillary tumor from 2 patients.

8.2.2 Mean Spectra and Ratio Spectra at 370 nm Excitation

The average curves were then made of the various diagnoses. The average curves do not provide an indication of the variation within a single diagnoses, so standard deviation lines were added for the benign mucosal tissue category only. Adding any more
standard deviation lines produced graphs so crowded they were hard to read. At 370 nm excitation, a generalization could be made that worsening disease caused a lowering of the overall amplitude such that benign mucosal tissue was the tallest, then dysplastic tissue, then CIS, then invasive cancer, and finally papillary tumors. (Fig. 8.3) Papillary tumors do not have a worse prognosis for the patient than invasive cancer or CIS, but it has a strongly different morphology which could account for its larger decrease in amplitude. The spread in the one standard deviation lines from the mean for benign mucosal tissue is much greater than the spread of the means for the various disease types. (Fig. 8.3) This suggests the peak value alone will not likely provide good diagnoses. At the 460 nm emission peak, dysplasia was 87% as tall as for benign mucosal tissue, CIS was 81%, invasive cancer was 69%, and papillary tumors were 56%. The 460 nm peak at one standard deviation below the mean for benign mucosal tissue is at only 17% of the height of the mean.

The ratio curves were taken with the various mean spectra for the disease types divided by that of the mean for benign mucosal tissue. (Fig. 8.4) Dysplastic tissue divided by benign mucosal tissue appears to the eye almost flat in the ratio graph, whereas CIS divided by benign mucosal tissue has a slight rise. Looking back at the average curves with the standard deviation, the standard deviation seems much larger than the averages. Invasive cancer has a more dramatic red rise with a 630 nm peak shape showing through. However, only a few of the invasive cancer had an obvious peak at this location and it seems less significant for diagnostic purposes due to its inconsistency. (Fig. 8.1) Dividing the 680 emission by the 600 emission provide a simple
measure of the red rise. At 370 nm excitation for benign mucosal tissue vs. papillary tumors, this results in 1.3 for the in vivo study and in 1.2 for the in vitro study. Thus, both the in vivo and in vitro provided similar waveshapes for papillary tumors.

For the one patient that had papillary tumors measured at 370 nm excitation, the average 460 nm emission peak was 1.8 times lower for the papillary tumors than for benign mucosal tissue. At 370 nm excitation for the cystoscopy study, the intensity is 2.6 lower at 460 nm emission in papillary tumors then in benign mucosal tissue. However, there was only one patient with papillary tumors measured at 370 nm excitation in the analysis for the in vitro study so caution is needed drawing conclusions based on that.

8.2.3 For Spectra Taken at 370 nm Excitation, Complications Within Tissue Diagnosed as Benign Mucosal Tissue

The pathologist reexamined most of the H&E stained slides of benign mucosal tissue and rated them for hemorrhage, edema, inflammation, and denuding. For the spectra taken at 370 nm excitation, twenty-eight sites had zero hemorrhage, 30 sites had category one hemorrhage, eight had category two hemorrhage, and none had category three hemorrhage. Thus, more benign mucosal sites had hemorrhage than did not. Plotting the averages for the different categories of hemorrhage at 370 nm excitation produced a dramatic change in overall amplitude of the 460 nm emission peak. (Fig. 8.5) With category one hemorrhage, the average peak is 49% as high as with no hemorrhage. With category two hemorrhage, the average peak is 43% as high as with no hemorrhage. This is more dramatic than the change in the 460 nm emission peak heights at 370 nm for

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the disease categories of dysplasia, CIS, invasive cancer, and papillary tumors. (Fig. 8.3)

Also of note with the average hemorrhage curves is the inclusion of the standard deviation lines for zero hemorrhage. (Fig. 8.5) They give a broader spread than the hemorrhage categories, indicating that hemorrhage alone does not account for the wide spread seen in the benign mucosal tissue category. In general, the more hemorrhage, the weaker the average signals.

The examination for edema as it corresponds to the spectra taken of benign mucosal tissue at 370 nm excitation revealed five sites with zero edema, six sites with category one edema, 36 sites with category two, and 19 sites with category three. Most of the benign mucosal sites had edema. There was no strong, obvious correlation between hemorrhage and edema. Plotting the averages for the different categories of edema produced a dramatic change in overall amplitude of the 460 nm emission peak. (Fig. 8.6) With category one edema, the average peak is 39% as high as with none; with category two, 53%; and with category three, 51%. This is also more dramatic than the change in the 460 nm emission peak heights at 370 nm for the disease categories of dysplasia, CIS, invasive cancer, and papillary tumors. (Fig. 8.3)

At 370 nm excitation for the benign mucosal tissue, there were seven sites with zero inflammation, 36 sites with category one inflammation, 21 sites with category two, and 2 sites with category three. There was no obvious correlation with either hemorrhage or inflammation. Plotting the averages for the different categories of inflammation
produced a dramatic change in overall amplitude of the 460 nm emission peak, but with no obvious trend as inflammation progressed since the 460 nm peak was largest for category one and weakest for category three. (Fig. 7)

At 370 nm excitation for the benign mucosal tissue, 12 sites had zero denuding, 11 sites had category one denuding, 31 sites had category two, and 12 sites had category three. Unlike the previous experiments, plotting the averages for the different categories of denuding did not produce as dramatic a change in overall amplitude of the 460 nm emission peak. (Fig. 8.8) With category one denuding, the average peak is 113% as high as with none; with category two, 74%; and with category three, 90%.

The great variation in the benign mucosal tissue which is caused by the hemorrhage, edema, and inflammation and perhaps other causes too makes it unlikely any diagnostic routine for finding diseases that cause less overall variation in the spectra will get excellent results. If there is some distinctive part of the spectra, PCA may work better at finding it since it uses the whole spectrum then taking only a selected emission wavelengths. With this much variation, though, there is too much likelihood of success.

8.2.4 Mean Spectra and Ratio Spectra at 400 nm Excitation

The bladder *in vivo* cystoscopy study found 400 nm excitation to produce moderately better results in sensitivity and specificity. Examining the average, standard deviation, and ratio curves for that may indicate may be more promising. As at 370 nm excitation, a generalization could be made that worsening disease caused a lowering of the overall amplitude such that benign mucosal tissue was the tallest, then dysplastic
tissue, then CIS, then invasive cancer, and finally papillary tumors. (Fig. 8.9) At the 460 nm emission peak, dysplasia was 94% as tall as for benign mucosal tissue, CIS was 71%, invasive cancer was 40%, and papillary tumors were 17%. This is a stronger change than at 370 nm excitation. However, even at 400 nm excitation, the 460 nm peak at one standard deviation below the mean for benign mucosal tissue is also much below the mean. It is at only 14% of the height of the mean. The variability in benign mucosal tissue is still great.

At 400 nm excitation, for the *in vitro* study the 460 nm emission peak, the intensity was 6.0 times lower for papillary tumors than benign mucosal tissue while for the *in vivo* study the intensity was 7.0 times lower. This agrees well for papillary tumors between the two studies.

For the 400 nm excitation mean spectra, the ratio curves were taken with the various mean spectra for the disease types divided by that of the mean for benign mucosal tissue. (Fig. 8.10) Dysplasia divided by benign mucosal tissue is a flat line indicating minimal change in spectral shape. All the other ratio curves have a red rise. The red rise for invasive cancer divided by benign mucosal tissue has in it sharp peaks at 630 and 680 nm suggesting porphyrins. [11] As shown earlier, these peaks were not a consistent effect for all the measured spectra for invasive cancer at 400 nm excitation and one spectrum with particularly high peaks greater than the mean plus three times the standard deviation for the spectra of all the other spectra diagnosed as invasive cancer was
removed from further analysis. Remaining after that spectrum is removed is another spectrum with large peaks at 630 and 680 nm that does not quite reach high enough to be excluded, but still is the largest contributor to the peaks seen in the ratio graph for the averages. (Fig. 8.2)

At 400 nm excitation for benign mucosal tissue vs. papillary tumors, dividing the 680 emission by the 600 emission results in 2.5 for the \textit{in vivo} study and in 1.6 for the \textit{in vitro} study. Thus, both the \textit{in vivo} and \textit{in vitro} provided somewhat similar waveshapes for papillary tumors although the \textit{in vivo} spectra had on average a more dramatic red rise.

Comparing to the \textit{in vivo} study, there the 460 nm emission peak was 6.3 times lower for papillary tumor then for benign mucosal tissue, while for this \textit{in vitro} study, the intensity is 6.0 times lower. This close agreement indicates that papillary tumors are responding similarly \textit{in vivo} and \textit{in vitro} at lease in regard to overall amplitude. However, out of the 30 patients used in the \textit{in vitro} analysis at 400 nm excitation, only two patients had papillary tumors while a majority of the patients in the \textit{in vivo} study had them. This is mainly an effect of the patient selection process with patients having bladders so diseased to require removal having few papillary tumors.

\textbf{8.2.5 For Spectra Taken at 400 nm Excitation, Complications Within Tissue Diagnosed as Benign Mucosal Tissue}

For the benign mucosal tissue spectra taken at 400 nm excitation, 29 sites had zero hemorrhage, 33 sites had category one hemorrhage, eight had category two hemorrhage, and none had category three hemorrhage. Thus, more benign mucosal sites
had hemorrhage than did not. Plotting the averages for the different categories of hemorrhage produced a dramatic change in overall amplitude of the 460 nm emission peak. (Fig. 8.11) With category one hemorrhage, the average emission peak at 460 nm is 46% as high as with no hemorrhage and with category two, 39% as high. One standard deviation below the mean for zero hemorrhage was only 26% as high for the 460 nm emission peak as for the mean. Thus, hemorrhage is not the only factor causing the large variation in the benign mucosal tissue fluorescence spectra at 400 nm excitation.

For the plot of ratio curves of category one and category two hemorrhage taken with respect to zero hemorrhage, the curves have the characteristic hemoglobin dips at 420, 530, and 580 nm with the dips stronger for the category two curve then the category one curve. (Fig. 8.12) [9, 10]

At 400 nm excitation for the benign mucosal tissue, there were seven sites with category one edema, 39 sites with category two, and 19 sites with category three. There was no strong and obvious correlation between hemorrhage and edema. Plotting the averages for the different categories of edema produced a dramatic change in overall amplitude of the 460 nm emission peak. (Fig. 8.13) With category one edema, the average peak is 108% as high as with none; with category two, 77%; and with category three, 49%. There seemed no obvious correlation with hemorrhage. The ratio curves were taken with respect to zero edema. (Fig. 8.14) While the ratio of category one edema to zero edema dropped off in the red, the other two ratios where fairly flat, although at amplitudes below 1.0.
At 400 nm excitation for the benign mucosal tissue, there were eight sites with zero inflammation, 39 sites with category one inflammation, 21 sites with category two, and 2 sites with category three. There was no obvious correlation with either hemorrhage or inflammation. Plotting the averages for the different categories of inflammation produced a dramatic change in overall amplitude of the 460 nm emission peak, but with no obvious trend as inflammation progressed since the 460 nm peak was largest for category one and weakest for category three. (Fig. 8.15) This is similar to the behavior for inflammation at 370 nm excitation.

At 400 nm excitation for the benign mucosal tissue, 15 sites had zero denuding, 12 sites had category one denuding, 31 sites had category two, and 12 sites had category three. Unlike the previous experiments at 400 nm excitation, plotting the averages for the different categories of denuding did not produce as dramatic a change in overall amplitude of the 460 nm emission peak. (Fig. 8.16) With category one denuding, the average peak is 91% as high as with none; with category two, 74%; and with category three, 75%. One standard deviation below the mean for zero denuding had a 460 nm emission peak at only 8% of that of the mean. This indicates most of the variation in benign mucosal tissue is not coming from denuding. This is supported by the ratio graphs which show dips at 420, 530, and 580 nm characteristic of hemoglobin absorption for the average of category three denuding divided by zero denuding. (Fig. 8.17) [9, 10] However, for the 12 sites with category three denuding, seven had category zero hemorrhage, 3 had category one, and two that category two, which does not indicate
significant hemorrhage. However, six of the twelve sites did have category two edema and the remaining six had category three edema, suggesting a correlation between edema and denuding rather than hemorrhage and denuding.

The great variation in the benign mucosal tissue which is caused by the hemorrhage, edema, and inflammation and perhaps other causes too makes it unlikely any diagnostic routine for finding diseases which in their average graphs cause considerably less overall variation in the spectra then these will get excellent results. If there is some distinctive shape within the spectra, PCA may work better at finding it since it uses the whole spectra then taking only a selected emission wavelengths. With this much variation in the benign mucosal tissue used as the category of non-diseased tissue, though, there is not too much likelihood of success.

### 8.2.6 Principal Component Analysis and Logistic Regression

#### 8.2.6.1 Benign Mucosal Tissue vs. Papillary Tumors

For the spectra taken at 400 nm excitation, the ROC curve for benign mucosal tissue vs. papillary tumors climbs up tightly into the upper left hand corner. (Fig. 8.18) There were 84 sites of benign mucosal tissue from 27 patients and only 6 sites of papillary tumors from 2 patients. Due to this unbalanced in sizes, when the PCA/LR method was run with a 50% probability decision threshold, the result was 67% sensitivity and 98% specificity. When the ROC curve is made by allowing the decision threshold to vary, much better sensitivity values can be seen on it with little loss in specificity. If the decision threshold was set to $84/(84+6)=93\%$ probability for separating non-diseased
from diseased categories, then the result is 100% sensitivity and 95% specificity. When the ROC curve is used instead and the point closest to (0,1) on it is chosen, the result is 100% sensitivity and 97% specificity.

This example highlights the effect set size can have on the final result. For papillary tumors, it is known that far more were observed in the in vivo study then in the in vitro study due to the patient selection process. Given this variability, the values from the ROC curve itself were taken which adjusted the threshold to optimize the trade-off between sensitivity and specificity rather than simply using a 50% probability decision threshold.

Four principal components were found significant below the 0.05 threshold for the pseudo $R^2$ value. (Fig. 8.19) Since the principal components are found before the procedure makes use of the pathologist's diagnoses, which come into play in the logistical regression step, these six principal components incorporate much of the variation in the 84 benign mucosal tissue spectra. While the principal components analysis is purely a mathematical technique that has not been specifically told the spectra of the chemical components within the tissue, sometimes values emerge which seem to come from specific chemical components because they cause much of the variance.

The first principal component has its main feature as the primary emission peak at 460 nm. The next diagnostically significant principal component, component three, has peaks at 420, 530, and 580 nm. Since the scores multiplied by the principal components can be positive or negative, a dip could mean a peak in the original spectra or vice versa. Thus, these seem the hemoglobin absorption dips. [9, 10] The next diagnostically
significant principal component, component five, has a peak around 510 nm. What that
could correspond to in the tissue does not immediately suggest itself. The next
diagnostically significant principal component, component six, has sharp dips at 530 and
580 nm, sharper than the peaks at 530 and 580 nm in principal component three.
(Fig. 8.19)

The PCA/LR method was not used at 370 nm excitation for benign mucosal tissue
vs. papillary tumors because there was only a single patient that had papillary tumors in
four sites in that analysis set. This seemed to small of a set to be meaningful.

For 400 nm excitation, as well as can be determined with only two patients in the
\textit{in vitro} study with papillary tumors, the sensitivity and specificity results after using the
ROC curves match well those observed in the \textit{in vivo} study. If there were more patients
with papillary tumors in the \textit{in vitro} study, the next step would be a leave-one-patient-out
cross-validation so that the reported sensitivity and specificity would provide diagnostic
potential since the algorithm would not see the data used to validate it. With only two
patients, that was not enough for that to have meaning.

\textbf{8.2.6.2 Benign Mucosal Tissue vs. All Diseases}

For the spectra taken at 400 nm excitation, the ROC curve for benign mucosal
tissue vs. all diseases including dysplasia, CIS, invasive cancer, and papillary tumors does
climb up above the 45° line of random chance. (Fig. 8.20) When the PCA/LR method
was run with a 50% probability decision threshold, the result was 81% sensitivity and
48% specificity. When the ROC curve is made by allowing the decision threshold to
vary, much better sensitivity values can be seen on it with little loss in specificity. If the
decision threshold was weighted to the sizes of the sets for non-diseased and diseased
categories, then the result is 79% sensitivity and 50% specificity. When the ROC curve is
used instead and the point closest to (0,1) on it is chosen, the result is 61% sensitivity and
96% specificity.

Three diagnostically significant principal components were found with pseudo R²
less than 0.05. (Fig. 8.21) The first, component one, has the 460 nm emission peak as its
main feature. The second, component three, has peaks at 420, 530, 580, and 630 nm. [9,
10, 11] It seems to have picked up the hemoglobin absorption dips with a bit of a
porphyrin peak mixed in. The third, component four, has sharp dips at 630 and 680 nm
which are almost certainly a result of porphyrin fluorescence. [11]

At 370 nm excitation, the results were not as good. With the 50% probability
decision threshold, the result was a 31% sensitivity and a 91% specificity. Weighting by
the set sizes changed this little with 33% sensitivity and 91% specificity. With the ROC
curve and finding the shortest distance to the (0,1) point, the result was a 71% sensitivity
and 52% specificity.

The sensitivity and specificity found from the ROC curves have the clearest
meaning for the purposes of this thesis. The 370 nm excitation results do not seem good
enough to encourage further analysis. The 400 nm excitation results are better with 61%
sensitivity and 96% specificity. However, the 61% sensitivity is not all that good, which
could improve by trading it off for some of the specificity. An examination of the ROC
curve reveals that the specificity drops off rapidly with little change in sensitivity.
When sensitivity and specificity are taken to be approximately equal, this gives a 68% sensitivity and 67% specificity. While better than random chance, it does not seem high enough to warrant continuing on with a leave-one-patient-out cross-validation. Instead, the diseases will be separated into different disease categories to uncover which are responsible for being clearly detected as diseased and which do not. The previous section demonstrated that papillary tumors were clearly detected, but are only a tiny part of the in vitro data set.

8.2.6.3 Benign Mucosal Tissue vs. Flat Diseases

What would be most clinically useful would be to detect the flat diseases of dysplasia and CIS. With 400 nm excitation, when the point closest to (0,1) on the ROC curve is chosen, the result is 60% sensitivity and 66% specificity. (Fig. 8.22) The ROC curve does not climb much from the 45° line of random chance. The principal components used seem to have the hemoglobin dips, and porphyrin values as described in previous sections. (Fig. 8.23) [9, 10, 11]

With 370 nm excitation, using the ROC curve results in 72% sensitivity and 57% specificity. When sensitivity and specificity are found on the curve when more approximately equal to each other, there was 60% sensitivity and 60% specificity. It does not seem better than the 400 nm excitation results.
8.2.6.4 Benign Mucosal Tissue vs. Dysplastic Tissue

To determine if dysplastic tissue or CIS different in their detectability, benign mucosal tissue was tested vs. each separately. With 400 nm excitation, when the point closest to (0,1) on the ROC curve is chosen, the result is 70% sensitivity and 51% specificity. (Fig. 8.24) The ROC curve does not climb much from the 45° line of random chance. There really were no principal component of diagnostic significance at or below the 0.05 level for pseudo RS. Principal component six with a pseudo $R^2$ of 0.29. The next smallest was principal component eight with a pseudo $R^2$ of 0.52. Only principal component six was used and it was used to get any diagnoses at all. (Fig. 8.25) The most obvious features in principal component six are three sharp dips at 460, 530, and 580 nm and sharp rises at 430 and 510 nm. It seems a mixture of hemoglobin and the main peak. [9, 10] As shown by the sensitivity and specificity, it did not work well at finding the diagnoses.

At 370 nm excitation, principal components three and seven both had pseudo $R^2$ less than 0.05. This suggested that 370 nm excitation might be better than 400 nm excitation for finding dysplasia since two principal components passed the significance test rather than none passing. However, when the point closest to (0,1) on the ROC curve is chose, the result is 71% sensitivity and 53% specificity. This is almost the same results as at 400 nm excitation.

Benign mucosal tissue and dysplastic tissue do not seem to separate well with fluorescence spectroscopy at either 370 or 400 nm excitation.
8.2.6.5 Benign Mucosal Tissue vs. CIS

With 400 nm excitation for benign mucosal tissue vs. CIS, when the point closest to (0,1) on the ROC curve is chosen, the result is 72% sensitivity and 63% specificity. (Fig. 8.26) This had two principal components with pseudo R2 under 0.05, components one and four. (Fig. 8.27) Principal component one had the 460 nm peak while principal component four has a peak at 430 and 550 nm and dips at 418, 460, and 630 nm. The 630 nm dip extends through 700 nm and would capture the red portion of the spectra.

At 370 nm excitation, using the ROC curve resulted in 65% sensitivity and 62% specificity, which is worse than the results at 400 nm excitation.

While benign mucosal tissue was more easily separated from CIS than dysplastic tissue, even the results for CIS are quite low at 72% sensitivity and 63% specificity. Leave-one-patient-out cross-validation was not done due to the low results when using the entire set of benign mucosal tissue and CIS.

8.2.6.6 Benign Mucosal Tissue vs. Invasive Cancer

With 400 nm excitation for benign mucosal tissue vs. invasive cancer, when the point closest to (0,1) on the ROC curve is chosen, the result is 82% sensitivity and 85% specificity. (Fig. 8.28) The ROC curve extends well out from the 45° line of random chance. Logistic regression determined two principal components had pseudo R2 under 0.05, components one and four. (Fig. 8.29) Principal component one features the primary emission peak at 460 nm. Principal component four has a dip around 440 and then two
very sharp and distinctive dips at 630 and 680 nm. The 630 and 680 nm dips are almost certainly due to porphyrin fluorescence, with a negative value for an associated score flipping a dip to a peak.

Benign mucosal tissue vs. invasive cancer finally gave results up over 80% for sensitivity and specificity. This is worth testing with leave-one-patient-out cross-validation to determine how diagnostically significant this really is. Fourteen patients that had invasive cancer with 38 invasive cancer sites. These same 14 patients had 28 benign mucosal tissue sites. Rather than using all the patients that had benign mucosal tissue but no invasive cancer, only patients that had invasive cancer too were used. Using all 14 patients with these 28 benign mucosal tissue sites and 38 invasive cancer sites as one group, the result was 93% sensitivity and 84% specificity. Next, the leave-one-patient-out cross-validation was done resulting in 79% sensitivity and 75% specificity.

With 370 nm excitation, using the ROC curve resulted in 88% sensitivity and 81% specificity, which is even slightly better than the results at 400 nm excitation.

Thirteen patients that had invasive cancer with 33 invasive cancer sites. These same 14 patients had 25 benign mucosal tissue sites. Using these 13 patients as one group, the result was 88% sensitivity and 92% specificity. Next, the leave-one-patient-out cross-validation was done resulting in 70% sensitivity and 80% specificity.

After performing the leave-one-out cross-validation, it seems fluorescence spectroscopy at either 370 or 400 nm excitation could have some utility at diagnosing
invasive cancer from benign mucosal tissue, although not with too high as sensitivity and
specificity. These were not the very high sensitivities and specificities found for
detecting papillary tumors in the *in vivo* study.

**8.2.7 Method of Using Only the 460 nm Emission Peak Height at 400 nm**

**Excitation**

One technique to find significant values within spectra it to take a t-test and look for p-values at 0.05 or under. This was done with a two-sided t-test assuming equal variance for the *in vitro* spectra at 400 nm excitation for the emission peak at 460 nm. A scatter plot of these 460 nm emission values shows a wide spread whatever the diagnosis, but there does seem a decreasing trend for more severe disease types. (Fig. 8.30) A plot showing the means and standard deviation makes this trend more visible. (Fig. 8.31) For benign mucosal tissue vs. the flat diseases of dysplasia and CIS, the result was 0.059, not quite passing the 0.05 or under test. Breaking this up into benign mucosal tissue vs. dysplasia and benign mucosal tissue vs. CIS, with dysplasia fails miserably with a p-value of 0.519 but CIS passes at 0.023. For benign mucosal tissue vs. invasive cancer, the p-value passes at a tiny $1.8 \times 10^{-5}$. For benign mucosal tissue vs. papillary tumors, the p-value passes at $8.6 \times 10^{-4}$. For benign mucosal tissue vs. all disease, the p-value passes at $7.0 \times 10^{-4}$.

As discussed in Chapter Four, a significant p-value only indicates a significant difference in the means, not how well the results will be for diagnostic purposes.
Slowly incrementing the threshold for discriminating between non-diseased and diseased classification to generate an ROC curve, 49% sensitivity and 56% specificity were found for benign mucosal tissue vs. flat diseases of dysplasia and CIS. Since that did fail the 0.05 test, although not by much, this poor sensitivity and specificity is not too surprising. For benign mucosal tissue vs. CIS, which did pass the p-value test, the result was 72% sensitivity and 52% specificity. (Fig. 8.32) For benign mucosal tissue vs. invasive cancer which passed the 0.05 p-value test with a minuscule 1.8x10⁻⁵, the result was 68% sensitivity and 74% specificity. (Fig. 8.33) These sensitivities and specificities are so small as unlikely to be diagnostically useful and are certainly much worse than the results over ninety percent that a literature search as described in Chapter One would have one expect. Furthermore, these results were generated using only one set without any validation procedure. Testing with a validation set generally only makes the sensitivity and specificity worse.

Anidjar et al. found exciting bladder tissue at 308 nm produced two emission peaks, one at 360 nm and one at 440 nm. They used the ratio of these peaks and reported excellent results. [1, 2, 3] In those same articles, though, they also used 337 nm excitation, then only using the emission peak height at 450 nm, reported finding bladder tumors had a p-value under 0.05. [1, 2, 3] For the studies in this thesis, the 360 nm emission could not be collected because it is a shorter wavelength then the excitations of 370 and 400 nm so Anidjar's ratio method for the two emission peaks could not be done. However, with 400 nm excitation, the emission peak in the in vitro study for this thesis is at about 460 nm. Other than a ten nm difference in peak location, using the 460 nm
emission peak to discriminate between the various tissue diagnoses would be the method
described by Anidjar. [1, 2, 3] Therefore, a two-sided t-test assuming equal variances
was preformed on the 460 nm emission values and the p-values found to be under 0.05
for benign mucosal tissue vs. CIS, benign mucosal tissue vs. invasive cancer, benign
mucosal tissue vs. papillary tumors, and benign mucosal tissue vs. all the disease
combined. However, the sensitivities and specificities in the table below corresponding
to these p-values are not high other than for benign mucosal tissue vs. papillary tumors.

| Benign vs. mucosal tissue vs. | p-value for 460 nm peak with peak with peak with PCA PCA |
|------------------------------|-----------------------------|-----------------------------|-----------------------------|
| flat disease                 | 0.059                       | 69%                         | 52%                         | 60%                         | 66%                         |
| dysplasia                    | 0.519                       | 65%                         | 52%                         | 70%                         | 51%                         |
| CIS                          | 0.023                       | 72%                         | 52%                         | 72%                         | 63%                         |
| invasive cancer              | \(1.8 \times 10^{-5}\)      | 68%                         | 74%                         | 82%                         | 85%                         |
| papillary tumor              | \(8.6 \times 10^{-4}\)      | 100%                        | 86%                         | 100%                        | 97%                         |
| all disease                  | \(7.0 \times 10^{-4}\)      | 58%                         | 74%                         | 61%                         | 96%                         |

Table 8.1: Measured p-values, sensitivities, and specificities.

In general, at 400 nm excitation, the PCA/LR method generated a better
combination of both sensitivity and specificity off the ROC curve then simply using the
peak height. The PCA/LR method has access to the rest of the spectral shape rather than
just the peak so should do better. For the in vitro experiment for this thesis, passing a p-
value under 0.05 did not show that a diagnostically useful sensitivity and specificity would necessarily result. This was true whether the method was simply using the peak height or the more sophisticated PCA/LR.

D'Hallewin et al. used fluorescence spectroscopy to detect bladder TCC and CIS without the use of exogenous dyes at excitations of 355 and 365 nm. [4, 5, 6, 7] D'Hallewin et al. reported 100% sensitivity and 100% specificity at 365 nm excitation, close to the 370 nm excitation used in this chapter, although D'Hallewin et al. used their entire data set without a validation set. [6] However, D'Hallewin et al. divided their diseased spectra by benign mucosal tissue, or normal tissue in their terminology, for each patient. [4, 6] This is the normalization to the patient's own benign mucosal tissue which deliberately was not done in this chapter because there was not an indication of what was benign mucosal tissue and what was dysplastic tissue and what was CIS until the biopsies were reviewed by a pathologist. Therefore, the method of D'Hallewin et al. was not used for this in vitro cystectomy data set.

For the same reason of avoiding that normalization procedure, the method of Cothren et al. for the colon using the 460, 600, and 680 nm emission values after normalizing to the patient's own benign mucosal tissue is also not reported for this bladder in vitro cystectomy data set. [8]

8.3 Conclusions

The physician could not grossly distinguish benign mucosal tissue from dysplasia or CIS, instead often indicating up hemorrhagic or inflamed tissue as the diseases when
often they were not. This inability to grossly have an indication of benign mucosal tissue led to leaving out the procedure of normalizing to the patient's own benign mucosal tissue.

A strong porphyrin fluorophore was occasionally observed for tissue diagnosed as invasive cancer, but for much of the other tissue with the same diagnosis was not observed. The inconsistency of this fluorophore made it difficult to deal with.

For this bladder in vitro data set, hemorrhage, edema, and inflammation all had a strong effect on the spectra size to an even greater extent for most cases then the diseases of dysplasia, CIS, and invasive cancer. As a result, the standard deviations on the average graphs were extremely broadly spaced. (Fig. 8.3 and 8.10) This effect greatly decreases the chance of getting good sensitivities and specificities for detecting the various disease categories.

The worsening disease categories on average decreased the overall amplitude of the spectra and measuring at the 460 nm emission peak found several of the disease categories to pass a t-test with a p-value under 0.05, but the sensitivity and specificity were nevertheless low.

The method of PCA/LR did perform better then simply using the peak height at 460 nm, although both performed poorly for all diseases except papillary tumors and invasive cancer.

Papillary tumors were rarely observed in the in vitro tissue in this study, making comparison to the many papillary tumors seen in the previous in vivo study difficult.
While papillary tumors did seem well discriminated for this *in vitro* study, the number found were so small that a cross-validation analysis was not performed as it would lack much meaning.

Invasive cancer was diagnosed from benign mucosal tissue after cross-validation with 79% sensitivity and 75% specificity at 400 nm excitation and with 70% sensitivity and 80% specificity at 370 nm excitation.
REFERENCES


Figure 8.1: Individual invasive cancer spectra at 370 nm excitation along with curve of average plus three times standard deviation used to determine outliers.
Figure 8.2: Individual invasive cancer spectra at 400 nm excitation along with curve of average plus three times standard deviation used to determine outliers.
Figure 8.3: Average fluorescence spectra curves at 370 nm excitation along with plus and minus one standard deviation curves from benign mucosal tissue.
Figure 8.4: Ratio curves for average spectra of various disease types divided by average spectrum for benign mucosal tissue all at 370 nm excitation.
Figure 8.5: Average bladder fluorescence spectra at 370 nm excitation of benign mucosal tissue categorized with degree of hemorrhage from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of hemorrhage.
Figure 8.6: Average bladder fluorescence spectra at 370 nm excitation of benign mucosal tissue categorized with degree of edema from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of edema.
Figure 8.7: Average bladder fluorescence spectra at 370 nm excitation of benign mucosal tissue categorized with degree of inflammation from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of inflammation.
Figure 8.8: Average bladder fluorescence spectra at 370 nm excitation of benign mucosal tissue categorized with degree of denuding from none to practically no epithelium on scale of zero to three along with plus and minus one standard deviation from zero degree of denuding.
Figure 8.9: Average fluorescence spectra curves at 400 nm excitation along with plus and minus one standard deviation curves from benign mucosal tissue.
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Figure 8.16: Average bladder fluorescence spectra at 400 nm excitation of benign mucosal tissue categorized with degree of denuding from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of denuding.
Figure 8.17: Average bladder fluorescence spectra at 400 nm excitation of benign mucosal tissue categorized with one, two, and three degree of denuding divided by average spectrum with no denuding.
benign mucosal tissue vs.
papillary tumors
400 nm excitation

Figure 8.18: ROC curve of benign mucosal tissue vs. papillary tumors after applying the PCA/LR method to the fluorescence measurements at 400 nm excitation.
benign mucosal tissue vs. papillary tumors
400 nm excitation

Figure 8.19: Diagnostically significant principal components for benign mucosal tissue vs. papillary tumors for the fluorescence measurements at 400 nm excitation.
Figure 8.20: ROC curve of benign mucosal tissue vs. all diseases including dysplasia, CIS, invasive cancer, and papillary tumors after applying the PCA/LR method to fluorescence measurements at 400 nm excitation.
benign mucosal tissue vs.
all disease
400 nm excitation

Figure 8.21: Diagnostically significant principal components for benign mucosal tissue vs. all diseases including dysplasia, CIS, invasive cancer, and papillary tumors for the fluorescence measurements at 400 nm excitation.
Figure 8.22: ROC curve of benign mucosal tissue vs. the flat diseases of dysplasia and CIS after applying the PCA/LR method to the fluorescence measurements at 400 nm excitation.
benign mucosal tissue vs.
flat disease
400 nm excitation

Figure 8.23: Diagnostically significant principal components for benign mucosal tissue vs. the flat diseases of dysplasia and CIS for the fluorescence measurements at 400 nm excitation.
benign mucosal tissue vs. dysplastic tissue
400 nm excitation

Figure 8.24: ROC curve of benign mucosal tissue vs. dysplastic tissue after applying the PCA/LR method to the fluorescence measurements at 400 nm excitation.
benign mucosal tissue vs. dysplastic tissue
400 nm excitation

Figure 8.25: Diagnostically significant principal component for benign mucosal tissue vs. dysplastic tissue for the fluorescence measurements at 400 nm excitation.
benign mucosal tissue vs. CIS
400 nm excitation

Figure 8.26: ROC curve of benign mucosal tissue vs. CIS after applying the PCA/LR method to the fluorescence measurements at 400 nm excitation.
Figure 8.27: Diagnostically significant principal components for benign mucosal tissue vs. CIS for the fluorescence measurements at 400 nm excitation.
Figure 8.28: ROC curve of benign mucosal tissue vs. invasive cancer after applying the PCA/LR method to the fluorescence measurements at 400 nm excitation.
benign mucosal tissue vs. invasive cancer
400 nm excitation

Figure 8.29: Diagnostically significant principal components for benign mucosal tissue vs. invasive cancer for the fluorescence measurements at 400 nm excitation.
Figure 8.30: Scatter plot of 460 nm emission peaks at 400 nm excitation divided into diagnostic categories.
Figure 8.31: Averages and standard deviations of 460 nm emission peaks at 400 nm excitation divided into diagnostic categories.
benign mucosal tissue vs. CIS
400 nm excitation

Figure 8.32: ROC curve of benign mucosal tissue vs. CIS after applying the 460 nm emission peak height method to the fluorescence measurements at 400 nm excitation.
benign mucosal tissue vs. invasive cancer
400 nm excitation

Figure 8.33: ROC curve of benign mucosal tissue vs. invasive cancer after applying the 460 nm emission peak height method to the fluorescence measurements at 400 nm excitation.
DETECTION OF EARLY CANCEROUS CHANGES AND CANCER IN BLADDER TISSUE BY AUTOFLUORESCENCE AND REFLECTANCE

Volume II

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

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1999

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Graduate Program in Biomedical Engineering
CHAPTER 9

REFLECTANCE SPECTROSCOPY

9.1 Materials and Methods Specific to Reflectance Spectroscopy

Measuring the white light reflectance spectra of tissue, which differs from fluorescence, may provide diagnostic information. [1, 2, 3, 4]

Zhengfang et al. performed a multiple linear regression analysis (MLR) on measured colon reflectance. They found adenomatous vs. hyperplastic polyps with 86% sensitivity and 72% specificity. They also distinguished neoplastic tissue, which includes both types of polyp and adenocarcinoma, from nonneoplastic tissue with 91% sensitivity and 78% specificity. [5] The principal component analysis followed by logistic regression method (PCA/LR) used in this chapter is a form of multiple linear analysis.

9.1.2 Experimental Setup

For taking white light reflectance measurements, a Xenon flashtube (EG&G flashtube model FX-249) was added to the fluorescence spectroscopy instrument. A coverslip, the type used on microscope slides, acts as a beam splitter to reflect and direct the light along the same path as the laser light which excites fluorescence for fluorescence spectroscopy. The probe tip was cut at a 15° angle to reduce direct reflectance because it
would not get transmitted down the fiber at that angle. Therefore, primarily diffuse reflectance would be measured. The flashlamp, like the nitrogen laser, was triggered at 10 Hz and the detector and detector software operated properly without any changes.

By making the simplifying assumptions that the tissue has a simple exponential decay law (Beer's Law), the measured reflectance is a function of the effective total absorption coefficient. [2] By taking the log base ten of the measured reflectance intensity after subtracting the background measurements and dividing by a background-subtracted reference converted the units to apparent absorbance. [1, 2, 6]

9.1.3 Koenig's Method of Analyzing Reflectance Spectra

The first step of Koenig's method of analyzing reflectance is to least-squares fit a line to the selected spectrum from 640 to 680 nm. [1] (Fig. 9.1) The vertical distance from that LSF line to the spectrum is taken at 555 and 577 nm. This finds peaks for oxidized and deoxidized hemoglobin. These 555 and 577 nm values are then put into a linear set of equations with reference spectra for oxidized and deoxidized hemoglobin. Koenig et al. found only the total amount of hemoglobin, summing the results of oxidized and deoxidized hemoglobin as determined by the solution of the linear equations, significantly changed in diseased tissue while the ratio of oxidized vs. deoxidized hemoglobin did not have significance. [1] Koenig's method for calculating the total amount of blood in the tissue based on reflectance spectroscopy was used for the measured spectra in this study for comparison to the results of the PCA/LR method.
9.1.4 Mourant’s Method of Analyzing Reflectance Spectra

Mourant et al. used the measured reflectance intensity spectra, after background subtraction and dividing by a background-subtracted reference. [4] With this, greater absorption decreases the signal height rather than increases it when in the absorbance as use by Koenig et al. [1, 4] Therefore, the presence of hemoglobin is seen as dips at 420, 530, and 580 nm rather than as peaks. Mourant et al. observed in their data set of reflectance spectra measured of bladder tissue that in the ultraviolet range, particularly from 330 through 370 nm, the slope varied considerably between non-malignant and malignant tissue. For their data set, non-malignant tissue consistently had a positive slope and malignant tissue had a negative slope. Using the sign of these slopes as a diagnostic criterion, they got 100% sensitivity and 97% specificity. [4] Like Koenig’s method, this method was also used for the measured spectra in this study for comparison to the results of the PCA/LR method. It differs in the part of the spectra examined with Mourant’s Method in the UV while Koenig’s Method in the visible spectral range. [1, 4]

For the reflectance spectra measured in the studies for this thesis, the spectra begin at 360 nm, so when applying Mourant’s method, the best that can be done is to determine the slope from 360 through 370 nm rather than from 330 through 370 nm. To find the slope, a least-squares-fit line was calculated for each spectrum from 360 through 370 nm and the slope of that line was used. (Fig. 9.2)
9.1.5 Principal Component Analysis With Logistic Regression Method

The principal component analysis with logistic regression method for reflectance was identical to that described in depth in Chapter Four and in the *in vivo* and *in vitro* bladder fluorescence chapters, Chapter Seven and Chapter Eight.

9.2 Results of Reflectance Spectroscopy

9.2.1 Number of Patients and Sites For Each Disease Category

Of the 35 cystectomy patients, the first six were done before the flashlamp assembly was added to the instrument, so reflectance spectra were taken for 29 of the cystectomy patients. One of these patients had sites with signet cell carcinoma, a type of cancer not included in this study, so that patient was excluded. Three other patients had spectra that went negative. Since light intensity cannot be negative, this indicates an error in the background signal being too high since the background signal had been subtracted off. Those three patients were excluded rather than simply the spectra that went negative since the same background is subtracted from all the reflectance spectra of a patient. Another patient had a weak and extremely jumpy signal in all its spectra between 360 nm and 380 nm so this patient was also excluded. This left 24 patients. One of these 24 patients had no benign mucosa sites measured, but the other sites fit into other of the diagnostic categories so that patient was still used. This resulted in 80 benign mucosal tissue sites from 23 patients; 21 dysplasia sites from 12 patients; 13 CIS sites from eight patients; 30 invasive cancer sites form 10 patients; and six papillary tumor sites from two patients.
For the *in vivo* study, five patients had reflectance spectra taken. As stated back in the Chapter Seven, 6 benign mucosal tissue sites from four of the patients and 8 papillary tumor sites from three of the patients were analyzed for benign mucosal tissue vs. papillary tumors. Although not included in that chapter's analysis, there were also two sites of dysplastic tissue from two patients and one CIS site. The dysplasia and CIS for the *in vivo* study will be looked at in this chapter so they can be compared with the dysplasia and CIS in the *in vitro* study.

**9.2.2 Mean Spectra and Ratio Spectra**

To be prepared for examining the reflectance spectra, an effect certain to be present in at least some of the spectra is hemoglobin absorption. When hemoglobin absorption is plotted in absorbance units so that a higher result means more absorption, several peaks are apparent. [6, 7] (Fig. 9.1) The results for hemoglobin differ slightly if the hemoglobin is oxidized or deoxidized. For oxidized hemoglobin, there are absorption peaks at 415, 530, and 580 nm; whereas for deoxidized hemoglobin, there are absorption peaks at 420 and 550 nm. [6, 7]

The log base ten of a measured reflectance intensity spectrum after background subtraction and division by the background subtracted barium sulfate reference spectrum converts the units to apparent absorbance where a higher signal amplitude means greater absorbance. [1, 6] For the *in vivo* study results, the mean reflectance spectra for papillary tumors was the highest with the greatest absorption, then CIS, then dysplasia, and finally benign mucosal tissue. (Fig. 9.4) The plot includes one standard deviation lines from the
mean for benign mucosal tissue. Dysplasia, CIS, and papillary tumors are all outside the standard deviation lines indicating the groups of spectra for the diseases all separate well from the benign mucosal tissue.

For the *in vitro* study results, all the mean spectra except invasive cancer were tightly grouped, all sitting on top of each other. (Fig. 9.5) The lines for one standard deviation from the mean for benign mucosal tissue have about five times as much spread as the mean spectra for the various disease types, with the exception being invasive cancer which was approximately one standard deviation from the mean for benign mucosal tissue. This indicates that other than invasive cancer, the other spectra for the disease category do not seem to group too differently than benign mucosal tissue.

The ratio spectra were then taken of the averages for the different disease types with respect to the average for benign mucosal tissue. (Fig. 9.6) The curves for the ratio for dysplasia and for CIS are both flat lines nearly at an amplitude of 1.0, indicating little difference from benign mucosal tissue in shape or amplitude. For the average papillary tumors divided by the average benign mucosal tissue spectra, the average if taken on average over the full spectral range would still be very close to 1.0, but there are distinctive dips at 420, 530, and 580 nm which are compensated by other areas of the spectrum being higher than 1.0. (Fig. 9.6) Since these dips are at the same locations for peaks seen in the example of hemoglobin absorption, this indicates papillary tumors seem to less hemoglobin in them than benign mucosal tissue. (Fig. 9.3) For the average invasive cancer tumors divided by the average benign mucosal tissue spectra, the average from 360 nm to about 450 nm is about 1.2 with one small peak rising above that at
420 nm, while after 450 nm the ratio graph declines nearly to a value of 1.0. (Fig. 9.6) This steady drop off from 450 nm through 685 nm can be considered a fall off in absorbance in the red part of the spectrum. Keeping in mind this is working in absorbance units, for reflectance intensity this would be a rise in the red part of the average invasive cancer spectrum compared to the average benign mucosal tissue spectrum. This could mean part of the red rise seen in the invasive cancer fluorescence spectra in Chapter Seven and Chapter Eight may come from absorptive effects rather than fluorescence effects. However, for the few invasive cancer fluorescence spectra with sharp peaks at 630 and 680 nm, those are still from porphyrin fluorescence and not due to changes in reflectance.

9.2.3 Koenig's Method of Analyzing Reflectance Spectra

A scatter plot was made after applying Koenig's method to the bladder in vivo study data. (Fig. 9.7) [1] The benign mucosal tissue grouped at a lower value then the diseased tissue, but the scatter plot highlights that the papillary tumors still have a wide spread in values. The single CIS value had a low amplitude, making it easily mistaken for benign mucosal tissue, but the two dysplasia sites were higher than the benign mucosal tissue.

For the ROC curve of benign mucosal tissue vs. papillary tumors, it clearly comes well off of the 45° line of random chance, yet it still does not get very close to the optimal value of (0,1). (Fig. 9.8) The result was 75% sensitivity and 83% specificity.
For the ROC curve of benign mucosal tissue vs. all disease including dysplasia, CIS, and papillary tumors, the ROC curve looks quite similar to before. (Fig. 9.9) The result was 73% sensitivity and 83% specificity.

In a couple of other articles by Koenig et al. on fluorescence rather than reflectance, the non-diseased category contained both benign mucosal tissue and dysplasia with the groups called malignant and non-malignant. [8, 9] In the reflectance article, dysplasia is included with the diseased group and the term used for all the tissue in the disease group is neoplastic. [1] With the set of in vivo bladder reflectance in this thesis, 56% sensitivity and 60% specificity result from using dysplasia in the non-diseased category, which is a much worse result than having it in the diseased category. Therefore, it makes sense to include dysplasia as diseased for our data as well as the results reported for Koenig et al.

A scatter plot was made after applying Koenig's method to the bladder in vivo study data. (Fig. 9.10) [1] Unlike with the in vivo study data, there is not clear grouping of benign mucosal tissue vs. diseased. CIS and papillary tumors seem to have slightly less hemoglobin than benign mucosal tissue, in contrast to diseased tissue having more hemoglobin in the in vivo study for this thesis and in the results reported by Koenig et al. [1] In the graph, benign mucosal tissue, dysplasia, and invasive cancer all have an extremely wide spread and the means appear roughly equivalent. (Fig. 9.5 and 9.10) Finding sensitivities and specificities with ROC curves bears out what the scatter plot indicated with Koenig's method not working well on the in vitro data set for this thesis. (Table 9.1)
Benign Mucosal Sensitivity Specificity
Tissue vs.
Papillary tumors 83% 35% (Fig. 9.11)
All diseases 52% 56% (Fig. 9.12)
Dysplasia and CIS 49% 56%
CIS 54% 56%
invasive cancer 63% 56% (Fig. 9.13)

Table 9.1: Koenig's Method on in vitro data set.

Thus, Koenig's method worked reasonably well on the in vivo data set for both detecting papillary tumors and all diseases, although that was a tiny set of only five patients. For the in vitro data set, Koenig's method consistently gave low sensitivities and specificities. If the only reflectance spectra taken for this thesis were in vitro, the conclusion drawn might have been the Koenig's method did not work. However, since this thesis does have a small set of reflectance spectra taken in vivo, instead the conclusion is Koenig's Method did work for our in vivo data set and not for our in vitro data set. Since the article by Koenig et al. states their method is specifically attempting to determine the amount of blood in the tissue, this could be a factor that has changed for our in vitro data set by the action of taking the bladder out of the body even though measured within twenty to thirty minutes after its removal.

9.2.4 Mourant's Method of Analyzing Reflectance Spectra

Mourant's Method calculated the slope of each measured reflectance intensity spectrum, after background subtraction and dividing by a background-subtracted
reference, from 330 through 370 nm. [4] For the spectra for this study, the slope was taken of a least-square-fit line from 360 through 370 nm since 360 nm marks the start of the measured spectra with the instrument used in the studies for this thesis. (Fig. 9.2) A scatter plot was made after applying Mourant's method to the bladder \textit{in vivo} study data. (Fig. 9.14) [4] The benign mucosal tissue definitely grouped at a higher value than the diseased tissue, but despite this grouping only two diseased sites, one CIS and one papillary tumor, had slopes that were actually negative. For benign mucosal tissue vs. papillary tumors, this produced 13% sensitivity and 100% specificity.

Because different detectors, probes, and so forth were used than Mourant's, it seems the measured slope might differ. Given the clear grouping seen, Mourant's Method was modified to use a movable decision threshold. Using this, an ROC curve can be generated. For benign mucosal tissue vs. papillary tumors resulted in 75% sensitivity and 83% specificity, the same percentages for Koenig's method. (Fig. 9.15) Using this modified method, taking benign mucosal tissue vs. all diseases resulted in 73% sensitivity and 100% specificity, which is better than the 73% sensitivity and 83% specificity for Koenig's Method. (Fig. 9.16) (See Table 9.2)

Therefore, this modified Mourant's Method worked as well as Koenig's Method for detecting papillary tumors and even better than Koenig's Method at finding all diseases lumped together.
For the modified Mourant Method when dysplasia is included in the non-diseased category for non-malignant vs. malignant, this resulted in 78% sensitivity and 70% specificity. Thus, as with Koenig's Method, it works better to have dysplasia in the diseased category.

While Koenig's Method did not work well for the in vitro data set, that method concentrated on detecting hemoglobin. Since Mourant's Method is calculated with UV wavelengths away from the main hemoglobin absorption dips, it might work better. The scatter plot after finding the slopes is not encouraging with wide spreads in the data without clear grouping. (Fig. 9.17)

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<td>Papillary tumors</td>
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While the modified Mourant Method did in general outperform Koenig's Method for sensitivity and specificity, the results still are not high with none of the tested groupings having both sensitivity and specificity over 80%.

These tests were done with the entire data set of the appropriate disease diagnoses being tested as one group, not with a validation set. If validation sets were used, it would
almost certainly reduce the sensitivity and specificity. As with Koenig’s Method, the modified Mourant Method worked well with the *in vivo* data, but not well with the *in vitro* data.

### 9.2.5 Principal Component Analysis With Logistic Regression

#### 9.2.5.1 Benign Mucosal Tissue vs. Papillary Tumors

For the *in vivo* data set, while using Koenig’s Method and modified Mourant’s Method both resulted in 75% sensitivity and 83% specificity, using PCA/LR resulted in 100% sensitivity and 100% specificity. There was one diagnostically significant principal component with pseudo R2 less than 0.05, Component One. (Fig. 9.18) It has a slight peak at 440 nm but otherwise is little more than a flat, constant signal. Since the sensitivity and specificity were as high as it is possible, a leave-one-patient-out cross-validation was performed on the four patients. This resulted in 75% sensitivity and 100% specificity. Therefore, PCA/LR Method worked quite well for finding papillary tumors in the *in vivo* data set. It would be worthwhile to take a larger set of *in vivo* reflectance spectra to see if this result continues.

For the *in vitro* data set, while using Koenig’s Method resulted in 83% sensitivity and 35% specificity and modified Mourant’s Method resulted in 83% sensitivity and 55% specificity, using the PCA/LR Method resulted in a much superior 100% sensitivity and 92% specificity. The ROC curve generated using PCA/LR tightly moves close to the ideal (0,1) point. (Fig. 9.19) With only two patients with papillary tumors in the *in vitro* reflectance data set, leave-one-patient-out cross-validation was not performed.
Two principal components had diagnostic significance with pseudo R2 less than 0.05, Components Four and Six. (Fig. 9.20) Principal Component Four has as its main feature a large and sharp peak at 420 nm. Principal Component Six has two large and sharp dips at 550 and 580 nm. These peaks and dips make it seem that hemoglobin is the main feature being found.

With only two diagnostically significant principal components used, a scatter plot of the scores corresponding to those components can be made as well as showing decision lines. (Fig. 9.21) The solid line shows the 50% probability decision line is not well placed because the large number of benign mucosal tissue sites, 80, compared to the relatively small number of papillary tumor sites, 6, causes the line to be placed where it much favors finding benign mucosal tissue at the expense of papillary tumors with 67% sensitivity and 99% specificity. Using a weighted decision line at 80/(80+6)=0.93=93% level balances out the sensitivity and specificity much better to 100% sensitivity and 92% specificity, the same values found optimally close to the (0,1) point on the ROC curve. The weighted decision line is drawn as a dashed line in the plot. (Fig. 9.21)

9.2.5.2 Benign Mucosal Tissue vs. All Diseases

For the in vitro data set, while using Koenig's Method resulted in 52% sensitivity and 56% specificity and modified Mourant's Method resulted in 63% sensitivity and 55% specificity, using the PCA/LR Method resulted in 58% sensitivity and 80% specificity for the point closest to the ideal (0,1) point on the ROC plot. (Fig. 9.22) Moving to a different point on the ROC point to balance out sensitivity and specificity rather than to
get mathematically closest to \((0,1)\) provides 63% sensitivity and 66% specificity. The ROC curve, while off the 45° line of random chance, does not climb close to the ideal \((0,1)\) point. (Fig. 9.22)

Principal Component One and Five were found diagnostically significant with pseudo R2 of less than 0.05. (Fig. 9.23) Principal Component One has peaks at 420, 540, and 580 nm, suggesting it is finding mainly hemoglobin. Principal Component Five has a peak at 400 and dips at 380, 430, 540, and 680 nm, with the largest at 430 nm. Perhaps the 400 nm peak and 430 nm dip for Principal Component Five when mixed with the 420 nm peak for Principal Component One is picking up oxidized and deoxidized hemoglobin.

9.2.5.3 Benign Mucosal Tissue vs. Flat Diseases

For the \textit{in vitro} data set, while using Koenig's Method resulted in 52% sensitivity and 56% specificity and modified Mourant's Method resulted in 54% sensitivity and 55% specificity, using the PCA/LR Method resulted in 58% sensitivity and 63% specificity.

9.2.5.4 Benign Mucosal Tissue vs. Dysplastic Tissue

For the \textit{in vitro} data set, using the PCA/LR Method resulted in 82% sensitivity and 51% specificity. The ROC curve illustrates that this is the closest point to the ideal \((0,1)\) point. (Fig. 9.24)
9.2.5.5 Benign Mucosal Tissue vs. CIS

For the in vitro data set, while using Koenig's Method resulted in 54% sensitivity and 56% specificity and modified Mourant's Method resulted in 69% sensitivity and 70% specificity, using the PCA/LR Method resulted in 62% sensitivity and 65% specificity.

9.2.5.6 Benign Mucosal Tissue vs. Invasive Cancer

For the in vitro data set, while using Koenig's Method resulted in 63% sensitivity and 56% specificity and modified Mourant's Method resulted in 55% sensitivity and 83% specificity, using the PCA/LR Method resulted in 83% sensitivity and 86% specificity. The ROC curve extends far out from the 45° line of random chance. (Fig. 9.25)

Reflectance spectroscopy found invasive cancer about as well as fluorescence at 370 and 400 nm excitation when the entire set of benign mucosal tissue and invasive cancer was used.

With reflectance, four principal components were found diagnostically significant, Components One, Five, Eight, and Nine. (Fig. 9.26) Mourant et al. wrote about how if Mie scattering theory is assumed with the tissue modeled as spheres in a medium with diseased tissue having different size spheres from non-diseased tissue, an oscillation will be introduced into the spectra. [4] For the significant principal components, there seems to be a shift in a peak from about 420 nm for Five, 410 nm for Eight, and 400 nm for Nine. (Fig. 9.26) There also seems to be a shift in a peak from about 550 nm for Five, 540 nm for Eight, and 540 nm for Nine. However, given the complex shapes of the principal components, this could be reading too much into them.
Ten patients had invasive cancer with 30 invasive cancer sites. Nine of these ten patients had 21 benign mucosal tissue sites with one patient having invasive cancer but not benign mucosal tissue. Rather than using all the patients that had benign mucosal tissue but no invasive cancer, only patients that had invasive cancer too were used. Using all 10 patients with these 21 benign mucosal tissue sites and 30 invasive cancer sites as one group, the result was 80% sensitivity and 86% specificity, which is close to what was calculated with the full set. Next, the leave-one-patient-out cross-validation was done resulting in 83% sensitivity and 52% specificity, a large reduction in specificity and slight gain in sensitivity.

The cross-validation resulted in the same sensitivity and specificity whether the decision line was at 50% probability or weighted for the relative sizes for the calibration set. Since the validation sets were small since they included spectra for only a single patient, generally the decision threshold could move within a wide range without changing the results for that patient. Thus, with leave-one-patient-out cross-validation and using only the calibration set to adjust the decision thresholds from the ROC curve or by weighting for the relatives sizes for non-diseased and diseased, little could be done to trade off sensitivity vs. specificity. This is unlike when the entire set is used at one time where sensitivity and specificity are easily traded off by adjusting the probability decision for what is non-diseased and diseased.

For the reflectance spectra, the leave-one-patient-out cross-validation resulted in 83% sensitivity and 52% specificity. This compares with the cross-validation for the fluorescence spectra taken at 400 nm excitation resulting in 79% sensitivity and 75%
specificity and with the cross-validation for the fluorescence spectra taken at 370 nm resulting in 70% sensitivity and 80% specificity. All look strongly significant even after the more strenuous cross-validation procedure caused them to diagnose rather than simply explain variation, but are not at the nearly 100% sensitivity and 100% specificity the bladder articles discussed in Chapter One of this thesis could lead one to expect.

9.2.6 Complications Within Tissue Diagnosed as Benign Mucosal Tissue for *In Vitro* Reflectance Spectroscopy

Given the results already seen of the hemorrhage, edema, and inflammation in the fluorescence spectra of benign mucosal tissue and knowing that hemoglobin causes light absorption, variation in the benign mucosal tissue spectra can be expected and was seen with the large standard deviation. (Fig. 9.5) [1]

The benign mucosal tissue was subdivided into having Category Zero hemorrhage with 27 sites, One with 22 sites, Two with 10 sites, or Three with one site where Category Zero meant no hemorrhage, One meant focal mild, Three meant marked diffuse, and Two meant between focal mild and diffuse. For the average curve, Category One hemorrhage was not markedly different than Category Zero, but both Category Two and Three were different with both having absorption peaks larger than one standard deviation above the mean for Category Zero hemorrhage. (Fig. 9.27) Taking the ratio of Category One, Two, and Three to Zero shows the expected peaks at 420, 530, and 580 nm. (Fig. 9.28)

The benign mucosal tissue was also subdivided into having Category Zero edema with 5 sites, One with 5 sites, Two with 35 sites, or Three with 15 sites where Category
Zero meant no edema, One meant focal mild, Two meant between 10% and 75%, and Three meant greater than 75%. (Fig. 9.29) Taking the ratio of Category One, Two, and Three to Category Zero shows that the typical hemoglobin absorption peaks of 420, 530, and 580 nm for Category One divided by Category Zero, but these were dips for Category Three divided by Category Zero while Category Two divided by Category Zero was almost a flat line at 1.0. (Fig. 9.30) This indicates more hemorrhage for Category One edema than for tissue without edema, but less hemorrhage for Category Three edema than for tissue without edema. Edema and hemorrhage are not strongly tied together.

The benign mucosal tissue was subdivided into having Category Zero inflammation with six sites, One with 28 sites, Two with 23 sites, or Three with 3 sites where Category Zero meant no inflammation, One meant focal mild with cluster of a few cells, Three meant marked diffuse, and Two meant more than a few clusters but less than marked diffuse. (Fig. 9.31) Taking the ratio of Category One, Two, and Three to Zero shows that the typical hemoglobin absorption peaks of 420, 530, and 580 nm for Category Three divided by Category Zero, but these were dips for Category One divided by Category Zero while Category Two divided by Category Zero was almost a flat line at 1.0. (Fig. 9.32)

The benign mucosal tissue was subdivided into having Category Zero with ten sites, One with nine sites, Two with 29 sites, or Three denuding with 12 sites where Category Zero meant no denuding, One meant less than 10%, Two meant between 10%
and 90%, and Three meant greater than 90%. (Fig. 9.33) Compared to one standard deviation away from Category Zero denuding, the mean spectra for the different categories group tightly together.

9.2.7 Fluorescence Divided by Reflectance Spectra

Wu et al. described a technique of dividing fluorescence spectra by their corresponding absorbance spectra. [10, 11] That should reduce the absorption effects on the fluorescence. This was performed, excluding dysplasia spectra which had an average close to that of benign mucosal tissue already. (Fig. 9.34) The most striking change for the average curves is that the benign mucosal tissue and the CIS curves sit almost on top of each other while the invasive cancer spectra has a much different shape. However, it has been learned that the variance is also important. The standard deviation curves are included. (Fig. 9.34) The spread got worse, not better.

Before going further with this technique, benign mucosal tissue spectra were separated into classifications of Category Zero, One, and Two hemorrhage as given before by the pathologist. (Fig. 9.35) If the absorbance is indeed reduced, there should be little spread between these mean curves. There still is, though, with 460 nm peak of the average Category Zero hemorrhage 1.6 times higher then that of tissue classified with Category One and 0.9 times higher than Category Two. With straight 400 nm excitation without dividing by a reflectance spectra, the 460 nm peak of the average of the classification Zero hemorrhage is 1.7 times higher then that of tissue with 1 hemorrhage and 2.1 times higher then that of 2 hemorrhage. The absorptive effects of hemoglobin or
other absorbers were not removed or significantly reduced for Category One hemorrhage. For Category Two, the 460 nm value is close to that of Category One, but at other wavelengths there is a great difference from a different waveshape. (Fig. 9.35) Given the variation remained in the spectra even after taking the ratio of fluorescence to reflectance, this investigation was not continued.

9.3 Conclusions

The results for the in vivo bladder study appeared to be greatly better than those for the in vitro bladder study, but it is hard to conclude too much from that since only five patients had reflectance spectroscopy taken in the in vivo study. Examination of the mean spectra indicated that benign mucosal tissue, dysplasia, and CIS were tightly grouped together especially compared to the standard deviation for the in vitro study which explains the poor sensitivity and specificity determined for finding dysplasia, CIS, or dysplasia and CIS together as diseased. For the in vivo study, though, the distinctions were larger with a steady and significant decrease in absorption for the mean spectra from papillary tumor to invasive cancer to CIS to dysplasia and finally to benign mucosal tissue. Unfortunately, the in vivo data set for reflectance was small so this might be partly due to the small set size.

The main peaks seen were always at 420, 530, and 580 nm, which are known absorption peaks for hemoglobin. Edema and hemorrhage were not closely correlated so some tissue had much edema and little hemorrhage or vice versa, while some had both.
Mourant's Method which examined the slope in the UV portion of the spectra performed better than Koenig's Method which estimated the blood in the tissue based on measurements at 555 and 577 nm. Therefore, significant diagnostic information is in the UV part of the spectrum. Using PCA/LR incorporated the entire available spectral range and it outperformed both Mourant's and Koenig's Method.

For benign tissue vs. invasive cancer, the significant principal components had peaks which jumped from 400 to 410 to 420 nm and from 540 to 550 nm, which might fit with the oscillation expected from Mie scattering if the cells or cell nuclei are assumed to be of different size.

Performing a leave-one-patient-out cross-validation for benign vs. invasive cancer dropped the results to 83% sensitivity and 52% specificity compared to 80% sensitivity and 86% specificity when the entire set was used at one time. This decrease in results with cross-validation, which is more meaningful for diagnostic purposes, was also seen when performing cross-validation with fluorescence spectroscopy.

Hemorrhage, edema, and inflammation all remained strong confounding factors, although denuding seemed to have little effect on the reflectance spectral measurements.

Dividing the fluorescence by the reflectance spectra did not significantly reduce the variance. Furthermore, the pathologist's diagnoses for hemorrhage when applied to the ratioed spectra for benign mucosal tissue still had the mean spectra with and without spectra changing amplitude considerably.
REFERENCES


Figure 9.1: Example of Koenig's least-squares-fit line to a reflectance spectrum with the line fitted to the region from 640 through 680 nm. [1]
Figure 9.2: Example of Mourant's least-squares-fit line to a reflectance spectrum with the line fitted to the region from 360 through 370 nm. [4]
Figure 9.3: Oxidized and deoxidized hemoglobin absorption curves. [7]
Figure 9.4: Average reflectance curves of \textit{in vivo} bladder measurements along with plus and minus one standard deviation curves from benign mucosal tissue.
Figure 9.5: Average reflectance curves of \textit{in vitro} bladder measurements along with plus and minus one standard deviation curves from benign mucosal tissue.
Figure 9.6: Ratio curves for average spectra of various disease types divided by average spectrum for benign mucosal tissue for the *in vitro* bladder study.
Figure 9.7: Koenig's reflectance method applied to the in vivo bladder reflectance measurements. [1]
benign mucosal tissue vs. papillary tumors

Figure 9.8: ROC curve of benign mucosal tissue vs. papillary tumors after applying Koenig's reflectance method and gradually incrementing the decision threshold for the in vivo bladder reflectance measurements. [1]
benign mucosal tissue vs. all disease

Figure 9.9: ROC curve of benign mucosal tissue vs. all diseases including dysplasia, CIS, and papillary tumors after applying Koenig's reflectance method to the in vivo bladder reflectance measurements. [1]
Figure 9.10: Koenig's reflectance method applied to the in vitro bladder reflectance measurements. [1]
Benign mucosal tissue vs. papillary tumors

Figure 9.11: ROC curve of benign mucosal tissue vs. papillary tumors after applying Koenig's reflectance method and gradually incrementing the decision threshold for the *in vitro* bladder reflectance measurements. [1]
Figure 9.12: ROC curve of benign mucosal tissue vs. all diseases including dysplasia, CIS, invasive cancer, and papillary tumors after applying Koenig's reflectance method to the \textit{in vitro} bladder reflectance measurements. [1]
benign mucosal tissue vs. invasive cancer

Figure 9.13: ROC curve of benign mucosal tissue vs. invasive cancer after applying Koenig's reflectance method to the \textit{in vitro} bladder reflectance measurements. [1]
Figure 9.14: Mourant's reflectance method applied to the \textit{in vivo} bladder reflectance measurements. [4]
benign mucosal tissue vs. papillary tumors

Figure 9.15: ROC curve of benign mucosal tissue vs. papillary tumors after applying Mourant’s reflectance method and gradually incrementing the decision threshold for the in vivo bladder reflectance measurements. [4]
Figure 9.16: ROC curve of non-malignant vs. malignant tissue after applying Mourant's reflectance method and gradually incrementing the decision threshold for the *in vivo* bladder reflectance measurements. [4]
Figure 9.17: Mourant's reflectance method applied to the in vitro bladder reflectance measurements. [4]
benign mucosal tissue vs. papillary tumors

Figure 9.18: Diagnostically significant principal components for benign mucosal tissue vs. papillary tumors for the \textit{in vivo} bladder reflectance measurements.
benign mucosal tissue vs. papillary tumors

Figure 9.19: ROC curve of benign mucosal tissue vs. papillary tumors after applying the PCA/LR method to the in vitro bladder reflectance measurements.
Figure 9.20: Diagnostically significant principal components for benign mucosal tissue vs. papillary tumors for the *in vitro* bladder reflectance measurements.
Figure 9.21: Binary decision plot for the scores for the two significant principal components for benign mucosal tissue vs. papillary tumors for the *in vitro* bladder reflectance measurements with one decision line not accounting for the relative size distributions between the two diagnoses and the weighted decision line adjusting for it.
Figure 9.22: ROC curve of benign mucosal tissue vs. all diseases including dysplasia, CIS, invasive cancer, and papillary tumors after applying the PCA/LR method to the in vitro bladder reflectance measurements.
Figure 9.23: Diagnostically significant principal components for benign mucosal tissue vs. all diseases including dysplasia, CIS, invasive cancer, and papillary tumors for the \textit{in vitro} bladder reflectance measurements.
benign mucosal tissue vs. dysplastic tissue

Figure 9.24: ROC curve of benign mucosal tissue vs. dysplastic tissue after applying the PCA/LR method to the in vitro bladder reflectance measurements.
Figure 9.25: ROC curve of benign mucosal tissue vs. invasive cancer after applying the PCA/LR method to the *in vitro* bladder reflectance measurements.
benign mucosal tissue vs. invasive cancer

Figure 9.26: Diagnostically significant principal components for benign mucosal tissue vs. invasive cancer for the *in vitro* bladder reflectance measurements.
Figure 9.27: Average *in vitro* bladder reflectance spectra of benign mucosal tissue categorized with degree of hemorrhage from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of hemorrhage.
Figure 9.28: Average in vitro bladder reflectance spectra of benign mucosal tissue categorized with one, two, and three degrees of hemorrhage divided by average spectrum with no hemorrhage.
Figure 9.29: Average in vitro bladder reflectance spectra of benign mucosal tissue categorized with degree of edema from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of edema.
Figure 9.30: Average in vitro bladder reflectance spectra of benign mucosal tissue categorized with one, two, and three degrees of edema divided by average spectrum with no edema.
Figure 9.31: Average *in vitro* bladder reflectance spectra of benign mucosal tissue categorized with degree of inflammation from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of inflammation.
Figure 9.32: Average *in vitro* bladder reflectance spectra of benign mucosal tissue categorized with one, two, and three degrees of inflammation divided by average spectrum with no inflammation.
Figure 9.33: Average *in vitro* bladder reflectance spectra of benign mucosal tissue categorized with degree of denuding from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of denuding.
Figure 9.34: Average in vitro bladder fluorescence spectra taken at 400 nm excitation divided by the reflectance spectra for the various disease categories along with plus and minus one standard deviation from benign mucosal tissue.
Figure 9.35: Average *in vitro* bladder fluorescence spectra taken at 400 nm excitation divided by the reflectance spectra for benign mucosal tissue categorized with degree of hemorrhage from none to saturated on scale of zero to three along with plus and minus one standard deviation from zero degree of hemorrhage.
10.1 Overview

For our studies, excitation wavelengths of 370 nm and 400 nm were chosen. This matches fairly closely the excitation maximums found by Audin for a variety of mammalian cells. Audin used a Zeiss microscope to image the autofluorescence and a Perkin-Elmer fluorescence spectrophotometer to take spectra of the autofluorescence of various mammalian cells with the labels of Chinese hamster ovary (CHO) SC1, CHO C14, CHO AB1, POS, B8, and 3T3B. Audin found excitation peaks at 360 nm and 400 nm. Audin credited the excitation peak at 360 nm along with an emission peak at 460 nm in the cells to NADH. Audin credited the excitation peak at 400 nm with an emission peak at 520 nm to riboflavin although riboflavin itself in phosphate-buffered solution (PBS) has an excitation peak at 460 nm, with a broad range though, with an emission peak still at 520 nm. For both excitations, the emission came from the cytoplasm and not from the cell nuclei. [1] Since the mammalian cells fluoresced well at these excitations, this suggested these two excitations might also work well for causing fluorescence in
bladder epithelial cells. Audin also found the autofluorescence of these cells increased as they entered exponential phases of growth. [1] This might be expected in fast-growing cancer cells.

The equipment used in our study was an Olympus microscope with an attached CCD camera as discussed in Chapter Three.

10.2 Colon Microscopy

The main purpose of this thesis is to detect early cancerous changes in bladder tissue by autofluorescence. It was based upon earlier spectroscopy results in the colon along with a preliminary study of taking microscope fluorescence images of colon tissue.

To better understand the fluorescence spectral results, the intrinsic fluorescence achieved without dyes (autofluorescence) has been characterized by fluorescence microscopy. As was discussed in Chapter One, various studies are investigating whether autofluorescence spectroscopy using an optical fiber probe inserted down the auxiliary channel of an endoscope can detect precancerous changes and cancer in the colon, bladder, and other organs. These studies have not reported including quantitative fluorescent microscopy to directly examine the fluorescing structures (fluorophores) within the tissue, with the exception of a study by Zonios et al. that used the same colon images collected for the preliminary study for this thesis. [2] This quantitative microscopy is important for understanding and characterizing the fluorescence rather than merely making educated guesses.
Frozen unstained sections have been imaged with a CCD camera on a fluorescence microscope to identify and reveal the morphology and location of the fluorophores. The CCD camera has an extremely linear response to light intensity, allowing images from it to be considerably more quantitative than acquiring images with standard film.

Analysis of the images identifies the fluorophores and maps them spatially. The microscope slides for the colon tissue were oriented on the microscope stage so that the horizontal axis represented depth into the tissue and the vertical axis was along the surface of the tissue. A hematoxylin and eosin (H&E) stained slide illustrates this, showing the lumen, mucosa, and submucosa. (Fig. 10.1) This image also illustrates the crypts lined with crypt cells, which are epithelial cells.

As was discussed in Chapter Two, a phase-contrast image is taken to display the tissue morphology. (Fig. 10.2) The crypts can be seen in this image. Without moving the slide, a fluorescence image was then taken. For normal colon tissue, the two fluorescing structures seem to be crosslinked collagen both in the submucosa and lamina propria fluorescing blue and inflammatory cells fluorescing yellow-green. (Fig. 10.2)

The images, taken with the microscope slide carefully oriented, are then integrated along the vertical axis to find the fluorescence density function of each fluorophore. (Fig. 10.3) There is fluorescence in this example image both in the mucosal and submucosal portions of the tissue. Since the crosslinked collagen appears as blue strings and what seems to be inflammatory cells appear as yellow-green spots, further processing
can use segment out the different fluorophores. [2] It was anticipated that analyzing bladder tissue imaged on the same equipment and with similar methods would provide similar results.

In the colon, there were not just two fluorophores observed, but three. The third is a broadband fluorescence in the cytoplasm of dysplastic crypt cells that intriguingly was not seen in the cytoplasm of normal crypt cells. [2] (Fig. 10.4) If the dysplastic or carcinoma-in-situ (CIS) cells in the bladder displayed a similar fluorescence, this could become a powerful diagnostic tool.

10.3 Methods Specific to Bladder Microscopy

10.3.1 Tissue Cut by Histology Technician

The colon study used samples frozen and sent from surgical pathology which were not the exact same specimens for which fluorescence spectra had been taken. For the bladder, tissue biopsies were taken as soon as measuring the fluorescence spectra was completed and the biopsies were immediately frozen in liquid nitrogen resulting in the spectra and microscopy being from precisely the same sites. The bladder tissue proved to be considerably harder to cut into nice sections than the colon tissue, so a trained histology technician cut the frozen tissue into 5 μm sections on a cryostat rather than a student or engineer. The slides were either used immediately or stored in a -80°C freezer.
10.3.2 Four Diagnostic Categories for Bladder Tissue

Serial sections were alternately immediately imaged on the fluorescence microscope or stained with hematoxylin-and-eosin (H&E). The H&E stained slides were later reviewed by a pathologist for his diagnoses. Rather than simply having diseased and non-diseased categories, the pathologist provided many more distinctions for the bladder tissue. This was simplified into categories of benign mucosal tissue, dysplasia, CIS, and invasive cancer.

10.3.3 Cube vs. Spherical Biopsy Shape

With the colon tissue, large frozen sections of the tissue had been provided from the pathology department. Cubes were cut with a forceps or razor blade. These were then oriented on blocks for cutting on the cryostat. The cube structure allowed easy orientation of the tissue such that one axis of the image would represent depth from the tissue surface. With the bladder tissue, the biopsies from the cup forceps formed the tissue into a ball of roughly 3 mm with much variation. The tissue was cut down to about halfway into the ball to orient the slices roughly perpendicular to the mucosal surface. For an analogy, consider slicing an orange through the middle to get approximately the true thickness of the skin while cutting at the ends could make the skin seem much too thick simply due to the angle of the cut. After cutting the tissue, the slide was rotated on the stage of the microscope so the horizontal axis of the image matched depth into the tissue and the vertical axis is parallel to the mucosal surface. This orientation was to allow later integration along the image's vertical axis to get a fluorescence density.
function based on depth in the tissue as had been done with the colon, but due to the
difference between the cube shapes of the colon biopsies and spherical shapes of the
bladder biopsies, the fluorescence density functions of the bladder will be more of an
approximation. [2]

Even when only considering collagen as a fluorophore since it was the strongest,
morphology including the thickness of the lamina propria with its lesser amount of
collagen compared to the greater and more densely packed amount in the deeper
submucosa also provided important diagnostic information.

Although Koenig et al. investigated autofluorescence of bladder tissue at 337 nm
excitation rather than the 370 and 400 nm excitations used for this thesis, their theory that
morphological changes as well as the type of fluorophores contribute to the emission
fluorescence needs to be considered too. [3] The tissue morphology affected measured
fluorescence spectra from the tissue surface for colon tissue. [2]

10.3.4 Both 380 nm and 400 nm Excitation

The earlier colon spectroscopy study had been at 370 nm excitation. Therefore,
the corresponding fluorescence microscopy was at 380 nm excitation, the closest
bandpass excitation filter available, to closely match the spectroscopy results. Both the in
vivo and in vitro bladder spectroscopy studies were done at both 370 nm and 400 nm
excitation. Therefore, for the bladder microscopy, the 75-W xenon arc lamp fitted with a
380 nm narrow-band interference filter for some images and 400 nm filter for others. At either excitation, the lens and a shutter still provided Kohler illumination at the microscopy slide with the tissue on the microscope stage. [4, 5, 6]

10.3.5 Exposure Time

Due to the thinness of the tissue, 5 μm, compared to an optical depth of roughly half a millimeter, long exposure times are needed for colon or bladder tissue. [2] The exposure time was adjusted to get to approximately half of its peak measurable intensity to take advantage of the 16-bit dynamic resolution to allow measurement of weak fluorescence effects unseen by the human eye. While the colon tissue required a 90 second exposure time to do this, the bladder tissue required 210 seconds. [2] This gives an indication of how much weaker the fluorescence seen in bladder tissue was then that seen in colon tissue.

10.3.6 Black-and-White and Color Fluorescence Images

Almost all of the colon images were collected only in black-and-white since the CCD camera is also in black-and-white. The promise of being able to segment the different fluorophores in the images using colors led to purchasing an additive red-green-blue filter set (Andover Corporation, Set 126FA44-25). Using these, three separate images could be collected and later combined into a single color image. Thus, for most of the bladder samples imaged on the microscope, most have a phase contrast image, a
black-and-white fluorescence image which has greater sensitivity to weak fluorescence since light is not blocked from the camera by colored filters, and three more images with the red, green, and blue filters respectively.

10.4 Results of Bladder Microscopy

Most of the microscope images were taken with 380 nm excitation, but since later analysis of the cystoscopy and cystectomy spectroscopy indicated 400 nm excitation was superior, images with 400 nm excitation were also taken.

10.4.1 Structure of Tissue

An H&E image of bladder tissue with benign mucosa shows the orientation arranged for the tissue for later calculation of the fluorescence density function. (Fig. 10.5) The horizontal axis represents depth into the tissue. To the left is the lumen of the bladder, followed by an epithelial cell layer which varies between 60 \( \mu m \) and 100 \( \mu m \) and then the submucosa. This image shows strong differences between bladder tissue and colon tissue. While the colon tissue had crypts lined by columnar epithelial and surrounded by lamina propria, the bladder tissue has a flat transitional cell epithelial layer without crypt structures but many cell layers thick. (Fig. 10.1 and 10.5)
10.4.2 Images Taken With 380 nm Excitation

10.4.2.1 Benign Mucosal Tissue

At 380 nm excitation for bladder tissue with benign mucosa, the phase-contrast image clearly demonstrates epithelial cells are present, but the corresponding fluorescence image displays nothing but black where the epithelium is at. (Fig. 10.6) This is with the sensitive black-and-white fluorescence image. A color image was constructed of images taken with the red, green, and blue filters. It demonstrates blue strands fluorescing in the submucosa, but also has no epithelial fluorescence. (Fig. 10.7)

10.4.2.2 Dysplastic Tissue

An H&E image of bladder tissue with dysplastic epithelium has an overall gross morphological appearance about similar to that of benign tissue. (Fig. 10.5 and 10.8) As with the benign mucosal tissue, the dysplastic epithelium produced no measurable fluorescence on the CCD camera with 380 nm excitation. (Fig. 10.9) The corresponding color image has only blue strands fluorescing in the submucosa without any epithelial fluorescence. (Fig. 10.10)

10.4.2.3 Carcinoma-In-Situ

As with both the benign mucosal tissue and the tissue with dysplastic epithelium, the layer where the CIS replaces the epithelium does not fluoresce with 380 nm excitation. (Fig. 10.11) The corresponding color image has only blue strands fluorescing in the submucosa. (Fig. 10.12)
10.4.2.4 Invasive Cancer

The H&E image of invasive cancer has a different appearance than that of the other tissue types because there is not epithelial layer covering it. (Fig. 10.13) All that is seen is invasive cancer. The fluorescence image at 380 nm excitation shows small fluorescing fragments within the cancer's bulk. (Fig. 10.14) The corresponding color image has blue and whitish-blue dots. (Fig. 10.15) The white portion comes from oversaturating. Thus, only the blue fluorophore seen before is apparent.

10.4.2.5 Fluorescence Density Functions

The black-and-white fluorescence images at 380 nm excitation were integrated along the vertical axis to produce the fluorescence density functions. (Fig. 10.16) It has 3 images from 2 patients averaged for the benign mucosal tissue, 2 images from one patient for the dysplastic tissue, 2 images from one patient for CIS, and 2 images from one patient for invasive cancer. Most noticeable is the lack of fluorescence shown in the fluorescence density function for approximately the first 100 μm. In other words, the epithelial cell layer shows no fluorescence in the epithelial layer in the images. The invasive cancer has sporadic places of small fluorescence with areas of almost no fluorescence. This fits with the consistently lower fluorescence seen in the invasive cancer spectra. The submucosa of both CIS and dysplasia is less than that of the submucosa of the benign mucosal tissues. The few number of images taken makes this more speculation than a definite result, but it seems the lowering in the intensity of the
average fluorescence spectra at 370 nm excitation of CIS when compared to benign mucosal tissue could be the result of changes in the submucosa rather than in the epithelial layer.

### 10.4.3 Images Taken With 400 nm Excitation

#### 10.4.3.1 Benign Mucosal Tissue

For benign mucosal tissue with 400 nm excitation, the epithelium also shows no fluorescence. (Fig. 10.17) The corresponding color image displays only blue strands similar to the results seen at 380 nm excitation. (Fig. 10.18) For this particular sample, the phase-contrast image shows a hole in the submucosa at the lower left. This could be a blood vessel, a lymph vessel, or an artifact from the cutting on the cryostat. For later calculation of the fluorescence density function, the part of the image with the hole was trimmed off. (Fig. 10.19 and 10.20)

#### 10.4.3.2 Proliferative Cystitis

Proliferative cystitis was observed. In one image, it shows a substantially thicker epithelium of approximately 250 to 300 μm. (Fig. 10.21) However, how much of this extra thickness is due to the epithelium actually being thicker and how much is due to the angle of the cut is uncertain. The corresponding black-and-white fluorescence image shows round spots fluorescing within the thick epithelial layer. (Fig. 10.22) The
corresponding color fluorescence image dramatically shows a yellow-green color to the fluorescing spots in the epithelium while the submucosa fluoresces blue. (Fig. 10.23)

This shows two of the fluorophores seen with the colon microscopy study.

A large cyst is also filled with epithelial cells as can be seen in an H&E image. (Fig. 10.24) The corresponding black-and-white fluorescence image shows small round dots fluorescing within the cyst. (Fig. 10.25) The corresponding color fluorescence image shows these dots to be yellow-green in color while the submucosa fluoresces blue. (Fig. 10.26) These yellow-green dots were only observed in this cyst and the proliferative cystitis with the thickened epithelium mentioned earlier, not in the other images.

10.4.3.3 Dysplastic Tissue

Only a small section of dysplasia was imaged at 400 nm excitation. The H&E image whose the small section. (Fig. 10.27) As at 380 nm excitation of dysplastic epithelium, the 400 nm excitation of dysplastic epithelium produces no fluorescence strong enough for the CCD camera to detect. (Fig. 10.28) The corresponding color fluorescence image has only blue fluorescence in the submucosa. (Fig. 10.29) For finding the fluorescence density function, this image was trimmed. (Fig. 10.30 and 10.31)
10.4.3.4 Invasive Cancer

The H&E image for the invasive cancer slide shows only the cancer cells. (Fig. 10.32) At 400 nm excitation, the black-and-white fluorescence image produces only small fluorescing fragments. (Fig. 10.33) The color fluorescence image has these fluorophores only producing blue light. (Fig. 10.34)

10.4.3.5 Fluorescence Density Functions

The fluorescence density functions were then produced from the images at 400 nm excitation. (Fig. 10.35) It uses 3 images from 2 patients averaged for the benign mucosal tissue, one image for the dysplastic tissue, no images for CIS, and 2 images from 2 patients for invasive cancer. Again noticeable is the lack of fluorescence shown in the fluorescence density function for approximately the first 100 μm. As before, the invasive cancer has sporadic places of small fluorescence with areas of almost no fluorescence which fits with the consistently lower fluorescence seen in the invasive cancer spectra. The submucosa of dysplasia is less than that of the submucosa of the benign mucosal tissues, but this is from only one dysplastic fluorescence image at 400 nm excitation so many not be a trend if other images of dysplastic tissue are taken.

10.4.4 Mercury Lamp with Color Film

Benign bladder tissue had been also imaged on with a Mercury lamp providing the excitation and with images taken on standard Kodak Ektachrome 200 speed color slide film. (Fig. 10.36 and 10.37) Unlike all the fluorescence images seen before, the
epithelium now demonstrates some fluorescence, although weaker then for the submucosa. Mercury lamps produce light in very narrow wavebands which has peaks at 254 nm, 297 nm, 313 nm, 365 nm, 405 nm, 436 nm, and so on for longer wavelengths. [7]

10.5 Discussion of Microscopy

10.5.1 No Epithelial Fluorescence at 380 nm and 400 nm Excitation

For both excitations, the fluorescence was dominated by blue fluorescence in the submucosa. At both 380 nm and 400 nm excitation, the images of bladder tissue covered by benign mucosa showed no epithelial fluorescence and only blue collagen strands in the submucosa. Dysplasia and carcinoma-in-situ (CIS) images appear grossly similar to the benign images in that the epithelial layer does not fluoresce and the submucosa does fluoresce in only blue strands. Thus, unlike the dysplastic crypt cells in adenomatous polyps of the colon which did show fluorescence at 380 nm excitation, the benign and dysplastic epithelial cells in the bladder as well as the epithelial cells converted to CIS do not show fluorescence at 380 nm and 400 nm excitation.

Despite the 16-bit dynamic resolution, the average intensity and variation in the part of the image with the epithelium matched that in the other part of the image with no tissue at all, just phosphate-buffer solution (PBS).
10.5.2 Inflammatory Cells as Second Rare Fluorophore in Bladder Tissue

An exception to having no fluorescence in the epithelium is a tissue sample that while benign mucosa is thicker with proliferative cystitis. In this thicker epithelium, which still appears black in the fluorescence images, there also appear bright yellow-green spots. These are thought to be inflammatory cells and were often seen in the colon tissue samples, but appeared much more rarely in the bladder tissue samples. This does provide a second fluorophore in bladder, but was rarely seen in the bladder microscopy.

10.5.3 Missing Porphyrin Fluorophore Sometimes Seen in Invasive Cancer

Spectra

Invasive cancer, which has no epithelial covering, also showed much less fluorescence in general. Fluorescence images of invasive cancer show collagen strands pushed aside and back by the cancer's bulk. This probably accounts for spectroscopically observed 3.2 times decrease of the 460 emission peak in the cystectomy study with 400 nm excitation for invasive cancer as compared to benign mucosal tissue.

The relative increase in the emission at 635 nm and 680 nm seen in the average invasive cancer spectra compared to the average benign mucosal tissue suggests another fluorophore which includes porphyrins. (See Chapter Eight) However, most of the fluorescence spectra for invasive cancer exhibited none to mild increase emission intensity at 635 nm and 680 nm but others had extremely intense peaks, on two occasions approaching the height of the 460 nm emission peak. The invasive cancer images taken did not provide that distinctive fluorophore. An explanation of this could be that
sometimes invasive cancer does have strongly fluorescing porphyrins, but often does not. The microscope images taken of invasive cancer just didn't happen to be the sites with the strongly fluorescing peaks at 635 nm and 680 nm.

10.5.4 Long Exposure Time

The exposure time was 90 seconds for colon tissue whereas it was 210 seconds for the bladder tissue images taken on the CCD camera. Both of these times were adjusted to get peak intensities of 30,000 to 40,000 to optimize the use of the 16-bit dynamic range. This much longer exposure time for bladder tissue indicates how much weaker it fluoresced. The collagen in the lamina propria of the colon tissue seemed far more densely packed than in bladder tissue. This is likely because the bladder is a much more distensible organ.

10.5.5 Thinning and Thickening of Bladder Epithelium

In the colon, the formation of a polyp involves extension of the crypts with increased replication of the crypt cells, a type of epithelial cell. In the bladder, thickening of the epithelium was rarely observed in the samples reviewed with the pathologist. Often, with dysplasia or CIS, there was rather extensive thinning and denuding instead. When thickening did occur, such as with proliferative cystitis, the pathologist's diagnosis was benign. This suggests the epithelial cell changes leading to a transformation to
cancer could be different for the bladder and colon, perhaps because of the structure the crypts provide in the colon or perhaps because the bladder epithelium is made of transitional cells.

10.5.6 Submucosal Fluorescence Differences

Since the amplitude of the reflectance at 460 nm provides statistically different grouping of CIS and invasive cancer from benign mucosal tissue, this strongly hints that some of the 460 nm emission amplitude difference seen with the fluorescence results from the light absorptive properties of the tissue. With 5 μm tissue sections, the distance is too short for much opportunity for the fluorescing light to be absorbed or scattered, so these fluorescence images provide practically no information on the light absorbers in the tissue, even though the absorbers or scatters could have important effects on the resultant emission at 460 nm as seen in the average fluorescence spectral curves for benign mucosal tissue, dysplasia, and CIS showing a steady decrease.

However, there was the intriguing lessening of intensity of intensity of the fluorescence in the submucosa for dysplasia and CIS compared to benign mucosa as seen in the fluorescence density functions. It could be as simple as a possibility of more swelling in the submucosa under areas of dysplasia or CIS in the epithelium, spreading apart the collagen fibers. It might also have some other explanation.
10.6 Summary

Against the expectations based on the fluorescence of dysplastic crypt cells in the colon, fluorescence in the epithelium of the bladder for benign mucosa, dysplasia, and CIS was not seen, even after switching from the reconstructed color images back to the more sensitive black-and-white images. The sparse and fragmentary fluorophores seen in the invasive cancer images explain the low overall fluorescence seen in the emission spectra, but do not explain the large emission peaks at 635 nm and 680 nm seen strongly in only a few invasive cancer spectra and hardly at all in the others. The fluorescence density images indicate there could be a change in the submucosa as shown by the lowering of its intensity for dysplasia and CIS.
REFERENCES


Figure 10.1: H&E microscope image of colon tissue with normal crypt cells.
Figure 10.2: Colon tissue with normal crypt cells as phase contrast microscope image to show tissue morphology and corresponding fluorescence microscope images at 380 nm excitation.
Figure 10.3: Fluorescence microscope image from Fig. 10.2 with its fluorescence density function.
Fig. 10.3: Integumentary Intensity (arb. units)

- Blue-white spots
- Yellow-green
- Overall fluorescence density function

- Submucosa
- Mucosa
- Lumen

- Blue crypt
- Yellow-green
Figure 10.4: Adenomatous colon tissue with dysplastic crypt cells as phase contrast microscope image to show tissue morphology and corresponding fluorescence image at 380 nm excitation.
Figure 10.5: H&E microscope image of bladder tissue with benign mucosa.
Figure 10.6: Bladder tissue with benign mucosa as black-and-white fluorescence microscope image at 380 nm excitation and corresponding phase-contrast image to show tissue morphology.
1 lumen-pj epithelium → submucosa

Figure 10.6
374
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Figure 10.10: Same tissue as Fig. 10.9 as color fluorescence microscope image at 380 nm excitation.
Figure 10.11: Bladder tissue with CIS as black-and-white fluorescence microscope image at 380 nm excitation and corresponding phase-contrast image to show tissue morphology.
Figure 10.11
Figure 10.12: Same tissue as Fig. 10.11 as color fluorescence microscope image at 380 nm excitation.
Figure 10.13: H&E microscope image of invasive cancer in bladder tissue.
Figure 10.14: Invasive cancer in bladder tissue as black-and-white fluorescence microscope image at 380 nm excitation and corresponding phase-contrast image to show tissue morphology.
Figure 10.14

lumen all invasive cancer
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Figure 10.16: Fluorescence density function at 380 nm excitation.
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Figure 10.18: Same tissue as Fig. 10.17 as color fluorescence microscope image at 400 nm excitation.
Figure 10.19: Same tissue as Fig. 10.17 as phase contrast microscope image showing how to trim image before calculating fluorescence density function.
Figure 10.20: Same tissue as Fig. 10.17 with same trimming as Fig. 10.19 as fluorescence microscope image at 400 nm excitation.
Figure 10.21: H&E microscope image of bladder tissue with benign mucosa, but also with proliferative cystitis.
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Figure 10.22
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Figure 10.25: Bladder tissue with benign mucosa with cystitis cystica as black-and-white fluorescence microscope image at 400 nm excitation and corresponding phase-contrast image to show tissue morphology.
Figure 10.25

large cyst (green dots in color fluor. Image)
Figure 10.26: Same tissue as Fig. 10.25 as color fluorescence microscope image at 400 nm excitation.
Figure 10.27: H&E microscope image of bladder tissue with dysplastic mucosa.
Figure 10.28: Bladder tissue with dysplastic mucosa as black-and-white fluorescence microscope image at 400 nm excitation and corresponding phase-contrast image to show tissue morphology.
Figure 10.28

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Figure 10.29: Same tissue as Fig. 10.28 as color fluorescence microscope image at 400 nm excitation.
Figure 10.30: Same tissue as Fig. 10.28 as phase contrast microscope image showing trimming of image before calculating fluorescence density function.
Figure 10.31: Same tissue as Fig. 10.28 with same trimming as Fig. 10.30 as fluorescence microscope image at 400 nm excitation.
Figure 10.32: H&E microscope image of invasive cancer in bladder tissue.
Figure 10.33: Invasive cancer in bladder tissue as black-and-white fluorescence microscope image at 400 nm excitation and corresponding phase-contrast image to show tissue morphology.
Figure 10.34: Same tissue as Fig. 10.33 as color fluorescence microscope image at 400 nm excitation.
estimated fluorescent density functions at 400 nm excitation

Figure 10.35: Fluorescence density function at 400 nm excitation.
Figure 10.36: H&E microscope image of bladder tissue with benign mucosa.
Figure 10.37: Bladder tissue with benign mucosa as color fluorescence microscope image with Mercury lamp excitation on Kodak Ektachrome 200 color camera film and corresponding Nomarski image to show tissue morphology.
CHAPTER 11

OVERALL CONCLUSIONS

11.1 Modeling of Bladder Tissue Fluorescence With Microscopy and Spectroscopy Results

For the colon, three fluorophores were observed under fluorescence microscopy: cross-linked collagen strands, inflammatory cells, and dysplastic epithelial cells. [1] By orienting frozen samples of the tissue, cutting them on a cryostat, and mounting the slices on microscope slides, black-and-white microscope images were taken with the horizontal axis providing depth into the tissue from the lumen and the vertical axis paralleling the lumen surface. Thresholding and masking techniques were performed on the images to separate the different fluorophores based on intensity with different images for each fluorophore. Integrating the images along the vertical axis created fluorescence density functions for each fluorophore. [1] These fluorescence density functions were a crucial component of the model of colon tissue fluorescence created by Zonios et al. They found inflammatory cells were not a significant cause of the fluorescence spectra measured from the probe, but the dysplastic epithelial cells and collagen were highly significant with the dysplastic epithelial cells providing diagnostic information. [1]
When excited at 370 nm, these fluorophores fluoresced at different colors. The collagen had emitted mainly with blue light, inflammatory cells with yellowish-green, and dysplastic crypt cells with a broadband gray. [1] The yellowish-green fluorescence could be the result of endogenous flavins which Benson et al. found occurred for a wide variety of cell types. [14] With the expectation of seeing similar fluorophores with similar color differences in bladder tissue, the quantitative microscopy with the cooled CCD camera was done in color by using a red, green, and blue filter separately. However, as discussed in Chapter Ten, the bladder proved to be a much different tissue than colon. While the colon tissue consistently had at least some inflammatory cells, the bladder tissue usually had none although there were exceptions. Thus, while for colon, Zonios et al. found inflammatory cells not a significant contributor to the measured fluorescence at the tissue surface, for bladder, this found inflammatory cells had even less effect since usually not even seen in the microscope images. The biggest change and surprise was finding no epithelial fluorescence in the bladder when excited at 370 or 400 nm regardless of whether the epithelium was benign, dysplastic, or carcinoma-in-situ. (Fig. 11.1 and 11.2)

For the fluorophores seen in the colon, for bladder tissue only the collagen was consistently present. It appeared in the submucosa as blue-colored strands. When the inflammatory cells did appear on a few of the microscope slide samples, they were yellowish-green in color as expected. The plan for pulling out different fluorophores in bladder tissue based on color did not work well because only one fluorophore, collagen, was seen in most of the microscope images. Unlike in the colon, the collagen was not tightly packed together. The bladder is a much more distendable organ than the colon and
must be more flexible. As a result of this decrease in amount of collagen packed together and overall amount in general, the exposure times for a fluorescent microscopy image of 90 seconds for colon tissue and of 210 seconds for bladder tissue produced approximately equal average intensities across the image.

As discussed in Chapter Eight, when bladder tissue became invasive cancer, occasionally strikingly strong porphyrin peaks at 630 and 680 nm were seen, but only stood out in about three of the 38 sites. (Fig. 11.3) This red fluorescence wasn't seen in the microscope images of bladder tissue with invasive cancer, but only three of the 38 sites had the strong peaks and those did not happen to be the ones with frozen samples taken and imaged.

Thus, for a model for bladder tissue fluorescence, there are three fluorophores but only one is consistently present. Fluorescing inflammatory cells were only seen in a few samples and were not seen in most samples. The strong porphyrin fluorescence was not seen in the microscope images, but was apparent in spectral measurements of three of the sites of invasive cancer. Collagen was the only fluorophore consistently present.

For benign mucosal tissue, dysplastic tissue, and CIS, there are two layers, an epithelium that does not fluoresce and a submucosa with collagen strands that do fluoresce. From the discussion of Chapter Six, the assumption is that for dysplasia and CIS, the changes are limited to the epithelium and the submucosa remains unchanged. This model would indicate benign mucosal tissue, dysplastic tissue, and CIS would all have identical fluorescence spectra. The diagnoses between these was indeed poor. Yet, the average curves at 400 nm excitation had a difference of intensity with benign mucosal
tissue the highest, then dysplastic tissue, and then CIS. (Fig. 11.4) Taking a t-test at the peak height of 460 nm as shown in Chapter Eight showed that CIS was different from benign mucosal tissue with a \( p=0.023 \) which is under 0.05. The fluorescence density functions from Chapter Ten indicate no epithelial fluorescence for benign mucosal tissue, dysplastic tissue, and CIS, but also that the submucosal fluorescence is weaker for dysplastic tissue and CIS then for benign mucosal tissue.

As reported in Chapter Eight, when the pathologist reviewed the benign mucosal tissue slides for the \textit{in vitro} study for edema and hemorrhage, most had some of both. Looking at the fluorescence spectra for benign mucosal tissue when using his diagnoses for hemorrhage in categories of zero through four had more variation in the mean then had been seen with benign mucosal tissue vs. the diseases of dysplasia and CIS. (Fig. 11.5) Taking the ratio of different categories of hemorrhage to no hemorrhage has absorption dips at 420, 530, and 580 nm, as expected for hemorrhage. (Fig. 11.6) [2, 3]

While edema also caused a large change in amplitude of the mean spectra of benign mucosal tissue at 370 and 400 nm excitation, the ratio of different categories of edema to no edema has no obvious absorption dips. (Fig. 11.7) Category One edema divided by Category Zero has a decreasing slope in the read while Category Three edema by Category Zero has an increasing slope. Therefore, edema seems to increase the absorption, but fairly uniformly.

When the ratio of measured reflected intensity, instead of absorbance units as in Chapter Nine, are used for benign mucosal tissue with different amounts of hemorrhage, the ratio of Category One, Two, and Three to Category Zero all show absorption dips at
420, 530, and 580, as expected. (Fig. 11.8) [2, 3] There is little absorption in the red portion of the spectra, which causes greater relative reflectance in the red. This causes a rise in the red portion of the spectra for the reflectance spectra. For the colon tissue, the red rise when mean diseased tissue was ratioed to mean non-diseased tissue rose above the ratio of 1.0, which indicates the presence of a fluorophore. For the bladder tissue, the red rise never rose above 1.0 and thus could partly or wholly be explained by absorptive effects except for the few fluorescence spectra for invasive cancer that clearly have distinct porphyrin fluorescence.

Thus, for benign mucosal bladder tissue, the model is an epithelial layer about 80 μm thick which does not fluorescence. Underneath that is a submucosa loosely packed with fluorescing collagen strands. Edema and hemorrhage is commonplace. Hemorrhage is known to have absorption peaks at 420, 530, and 580 nm. [2, 3] Increased edema was found to increase absorption too, but not with obvious peaks. Swelling of the tissue caused by it being filled with hemorrhage and edema could also decrease the relative amount of collagen present since the bladder is a highly distendable organ, which could reduce measured fluorescence.

It has been speculated in the literature that a strong fluorophore in bladder fluorescence would be NADH, which has an emission peak around 460 nm as is the peak observed for bladder tissue too. Collagen fluorescence has an emission peak around 390 nm, but that is when it is excited at its excitation peak of around 308 nm. [4] Excitation-Emission Matrix (EEM) measurements of collagen as well as literature using other excitations indicate a shift in the emission peak as the excitation peak changes so that
when excited at 370 to 400 nm, the emission peak will be in the range of 440-460 nm, as was observed. Technically, it is cross-linking in the collagen rather than just collagen causing the fluorescence.

Without any observed epithelial fluorescence, what changes was observed in CIS and dysplastic tissue likely comes from differences in the submucosa caused by edema and hemorrhage. This explains the large variance for benign mucosal tissue, dysplastic tissue, and CIS and their poor diagnostic differentiation yet also explains the difference that is seen in the t-test and mean graphs.

Invasive cancer invades the tissue and displaces most of the collagen although fragments are left behind. This is the reason for the low and sporadic fluorescence seen in the fluorescence density functions. (Fig. 11.1 and 11.2) Thus, the model for invasive cancer is a nonfluorescing media with small fluorescing bits of collagen scattered within it.

Due to seeing only two patients with papillary tumors out of the 35 *in vitro* bladder patients, frozen papillary tumor biopsies were not available for microscopic imaging. Chapter Six discussed bladder diseases and how papillary have a much different morphology then flat bladder wall. These papillary tumors grow out of the bladder wall like a tree. The submucosa for them is a blood vessel shaft running up the structure and not the same standard submucosa of flat bladder wall. The epithelium covering the papillary tumor is generally thicker than that of flat bladder wall. A thicker epithelium would decrease the returned signal as it increases the distance to the submucosa. The fluorescence underneath the epithelium in papillary tumors might have
different fluorophores which might increase red fluorescence or else since the ratio graphs
did not climb above one, there may be absorbers in the epithelium and/or submucosa that
have little absorption of red light.

As can be seen in Table 11.1, for fluorescence spectroscopy without a validation
set, only benign mucosal tissue vs. invasive cancer and benign mucosal tissue vs.
papillary tumors had sensitivity and specificity both about 80%. There were only two
patients in the in vitro study with papillary tumors.

<table>
<thead>
<tr>
<th>Benign Mucosal Tissue vs.</th>
<th>Sensitivity of 370 nm Excitation</th>
<th>Specificity of 370 nm Excitation</th>
<th>Sensitivity of 400 nm Excitation</th>
<th>Specificity of 400 nm Excitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary tumors</td>
<td>--</td>
<td>--</td>
<td>100%</td>
<td>97%</td>
</tr>
<tr>
<td>All diseases</td>
<td>71%</td>
<td>52%</td>
<td>61%</td>
<td>96%</td>
</tr>
<tr>
<td>Dysplasia and CIS</td>
<td>72%</td>
<td>57%</td>
<td>60%</td>
<td>66%</td>
</tr>
<tr>
<td>CIS</td>
<td>65%</td>
<td>62%</td>
<td>72%</td>
<td>63%</td>
</tr>
<tr>
<td>invasive cancer</td>
<td>88%</td>
<td>81%</td>
<td>82%</td>
<td>85%</td>
</tr>
</tbody>
</table>

Table 11.1: PCA/LR Method on *in vitro* fluorescence data set.

As can be seen from Table 11.2, when a validation set was not used, reflectance
spectroscopy did almost as well as fluorescence spectroscopy in terms of sensitivity and
specificity. This fits with the assumption for the model of only cross-linked collagen as a
fluorophore with most of the effects observed for changes in fluorescence spectra for
different diseases instead being changes in absorptive properties of the tissue.
### Table 11.2: PCA/LR Method on *in vitro* 400 nm excitation fluorescence and reflectance data sets.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sensitivity (Reflectance)</th>
<th>Specificity (Reflectance)</th>
<th>Sensitivity (400 nm Excitation)</th>
<th>Specificity (400 nm Excitation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary tumors</td>
<td>100%</td>
<td>92%</td>
<td>100%</td>
<td>97%</td>
</tr>
<tr>
<td>All diseases</td>
<td>58%</td>
<td>80%</td>
<td>61%</td>
<td>96%</td>
</tr>
<tr>
<td>Dysplasia and CIS</td>
<td>58%</td>
<td>63%</td>
<td>60%</td>
<td>66%</td>
</tr>
<tr>
<td>CIS</td>
<td>62%</td>
<td>65%</td>
<td>72%</td>
<td>63%</td>
</tr>
<tr>
<td>Invasive cancer</td>
<td>83%</td>
<td>86%</td>
<td>82%</td>
<td>85%</td>
</tr>
</tbody>
</table>

When leave-one-patient-out cross-validation was performed for the benign mucosal tissue vs. invasive cancer *in vitro* data set, the sensitivity and specificity decrease from when the data was processed without a validation set as can be seen in Table 11.3. While there does seem a difference between invasive cancer and benign mucosal tissue, it is not at the 80% and above levels of both sensitivity and specificity for finding papillary tumors as seen in Table 11.4.

### Table 11.3: Leave-one-patient-out cross-validation on *in vitro* data set.

<table>
<thead>
<tr>
<th>Benign Mucosal Tissue vs. Invasive Cancer</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>370 nm Excitation</td>
<td>70%</td>
<td>80%</td>
</tr>
<tr>
<td>400 nm Excitation</td>
<td>79%</td>
<td>75%</td>
</tr>
<tr>
<td>Reflectance</td>
<td>83%</td>
<td>52%</td>
</tr>
</tbody>
</table>

422
The papillary tumors come out from the flat bladder wall with a tree-like structure. This drastic change in tissue morphology along with a thickened epithelium causes most of or all of the change seen in fluorescence spectroscopy.

<table>
<thead>
<tr>
<th>Benign Mucosal Tissue vs. Papillary Tumors</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>370 nm Excitation</td>
<td>88%</td>
<td>91%</td>
</tr>
<tr>
<td>400 nm Excitation</td>
<td>82%</td>
<td>96%</td>
</tr>
</tbody>
</table>

Table 11.4: Leave-one-patient-out cross-validation on *in vivo* data set.

### 11.2 Future Work

#### 11.2.1 Shorter Excitation Wavelengths

Koenig with excitation at 337 nm and Anidjar with excitation at 308 nm reported another fluorophore at around 380 nm, unobservable with the excitations at 370 or 400 nm. [4, 9, 10, 11, 12] Running the nitrogen laser at 337 nm on bladder tissue did cause two peaks with one at about 380 nm, as they reported. Investigating at a shorter wavelength such as 337 nm, or at even shorter wavelengths of a different laser other than nitrogen is available, could be valuable since another fluorophore does seem to be present at those excitations.
11.2.2 Excitation-Emission Matrix Measurements of Tissue

Technology has been developed to measure the excitation-emission matrix (EEM) for tissue with a dozen excitations being used in less time than the three (370, 400 nm, and white light reflectance) used in the studies reported here. Other excitations potentially could reveal other fluorophores.

11.2.3 Alter Biopsy Procedure for Better Orientation

The biopsies for these studies were taken with cup biopsy forceps. The tissue is formed into a ball. This can cause problems with orientation of microscope images with one direction indicating depth into the bladder wall. Earlier with the colon tissue imaged under the microscope, small cubes of tissue were cut from the colon wall. These could be cut and oriented so depth into the tissue was easier to determine. A biopsy procedure that did this for bladder tissue would improve determining orientation.

11.2.4 In Vivo and In Vitro Measurements of Identical Bladders

One weakness of the in vivo and in vitro spectra in these studies was the different patient selection criteria caused a situation with much different disease profiles. The in vivo study primarily had papillary tumors as the frequently observed disease while the in vitro study had dysplasia, CIS, and invasive cancer as the frequently observed disease with only rare occurrences of papillary tumors. This makes comparison of the in vivo and in vitro results problematic. If in vivo spectra could be taken on a patient prior to a cystectomy operation and then on the removed bladder, it would answer the question of
whether and how much the fluorescence changes from in vivo conditions to in vitro after the bladder is removed from the body about twenty minutes.

11.2.5 Perfusion of In Vitro Bladder

The pathologist's diagnoses of hemorrhage within benign mucosal tissue when applied to the measured spectra indicated hemorrhage caused considerable variation in the measured spectra. An organ outside the body does not have the same perfusion of blood as an organ in the body. The blood pools inside a dead body and would do the same in an organ. Perfusion of the removed organ may offset this.

11.2.6 Seek Less Diseased In Vitro Bladder Tissue

The bladders in the in vitro study were removed because of cancer. As a result, the bladders were generally in bad condition which was the reason they were removed in the first place. Investigating less diseased bladders, perhaps harvested at autopsy from accident victims might better represent the condition of bladders seen in the in vivo study.

11.2.7 Fluorescence Confocal Microscopy

The microscopy images were taken with a conventional inverted fluorescence microscope. The tissue was cut into 5 μm sections to get excellent images. If the tissue had not been cut thin, the images would have been blurred by the many cells. However, the typical epithelial cells are roughly 10 μm in diameter so would be sliced open by being cut at 5 μm. It is conceivable that being sliced open could change the fluorescence
characteristics of the cells. Since the cells will not be perfectly aligned, the thickness would have to be greater than 10 \( \mu m \) to avoid cutting open most of the cells, but then the images would be blurry. A confocal microscope uses a focused beam and raster scanning so can take images of thick tissue yet not be blurry. Thus, imaging thicker specimens of bladder tissue with a confocal microscope could provide sharp, clear images without the cells being sliced open.

11.2.8 Scanning or Imaging System

Provided the fluorescence spectroscopy can be made more reliable for detecting diseases such as CIS, then it would be valuable to be able to sample tissue more quickly and over a greater area than provided by an optical fiber probe approximately one millimeter in diameter. Imaging the whole bladder would be the best. Such a system has been developed by Koenig et al. for use with fluorescence dyes. [13]

If that was unworkable for an autofluorescence system, then having a method to rapidly scan the probe over the tissue could also allow much more tissue to be examined more quickly.

11.2.9 Animal Study

Given the lack of availability of the patients for doing both the \textit{in vivo} and \textit{in vitro} studies for comparison of the same patient and the severe diseased nature of the removed bladders in the \textit{in vitro} study, an animal study might be worthwhile. If bladder cancer
could be induces in the bladders of rabbits or some other animal, this would allow
investigation at various stages of the disease and both in vivo and in vitro on the same
bladder.
REFERENCES


estimated fluorescent density functions at 380 nm excitation

Figure 11.1: Fluorescence density functions at 380 nm excitation.
estimated fluorescent density functions at 400 nm excitation

Figure 11.2: Fluorescence density function at 400 nm excitation
Figure 11.3: Individual invasive cancer spectra of *in vitro* bladder tissue at 400 nm excitation.
Figure 11.4: Average fluorescence density spectra curves of \textit{in vitro} bladder tissue at 400 nm excitation along with plus and minus one standard deviation curves from benign mucosal tissue.
Figure 11.5: Average bladder fluorescence spectra at 400 nm excitation of benign mucosal spectra categorized with degree of hemorrhage from none to saturated on a scale of zero to three along with plus and minus one standard deviation from zero degree of hemorrhage.
Figure 11.6: Average bladder fluorescence spectra at 400 nm excitation of benign mucosal spectra categorized with one, two, and three degree of hemorrhage divided by average spectrum with no hemorrhage.
Figure 11.7: Average bladder fluorescence spectra at 400 nm excitation of benign mucosal tissue categorized with one, two, and three degree of edema divided by average spectrum with no edema.
Figure 11.8: Average *in vitro* bladder reflectance spectra in measured intensity units, of benign mucosal tissue categorized with one, two, and three degree of hemorrhage divided by average spectrum with no hemorrhage.


