AUTOFLUORESCENCE-BASED DIAGNOSTIC UV IMAGING
OF TISSUES AND CELLS

by

Timothy E. Renkoski

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DEDICATION

I dedicate this work to my wife Chen and our parents Bartholomew and Elizabeth and Deqiang and Lin. Their love and support have been vital to its completion.
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ABSTRACT

Cancer is the second leading cause of death in the United States, and its early diagnosis is critical to improving treatment options and patient outcomes. In autofluorescence (AF) imaging, light of controlled wavelengths is projected onto tissue, absorbed by specific molecules, and re-emitted at longer wavelengths. Images of re-emitted light are used together with spectral information to infer tissue functional information and diagnosis.

This dissertation describes AF imaging studies of three different organs using data collected from fresh human surgical specimens. In the ovary study, illumination was at 365 nm, and images were captured at 8 emission wavelengths. Measurements from a multispectral imaging system and fiber optic probe were used to map tissue diagnosis at every image pixel. For the colon and pancreas studies, instrumentation was developed extending AF imaging capability to sub-300 nm excitation. Images excited in the deep UV revealed tryptophan and protein content which are believed to change with disease state. Several excitation wavelength bands from 280 nm to 440 nm were investigated. Microscopic AF images collected in the pancreas study included both cultured and primary cells.

Several findings are reported. A method of transforming fiber optic probe spectra for direct comparison with imager spectra was devised. Normalization of AF data by green reflectance data was found useful in correcting hemoglobin absorption. Ratio images, both AF and reflectance, were formulated to highlight growths in the colon. Novel tryptophan AF images were found less useful for colon diagnostics than the new ratio techniques. Microscopic tryptophan AF images produce useful visualization of cellular protein content, but their diagnostic value requires further study.
CHAPTER 1: INTRODUCTION

1.1 Motivation

Cancer is a devastating disease in which some cells of the body fail to regulate their growth and replicate unchecked leading to formation of masses or tumors. Such growths can metastasize, spreading throughout the body, and interfere with the function of vital organs leading to early death. Aggressive treatment of cancer by tissue resection followed by radiation therapy or chemotherapy is quite effective in curing early stage disease but much less effective in late stage disease. Consider, for example, that the five year survival chance of a person diagnosed with early localized colon cancer is currently 90%, but only 70% for a person diagnosed in middle stage, and just 12% for a person diagnosed after colon cancer has spread to distant organs. This example illustrates a general trend that applies not only to colon cancer but cancer in many other organs. Therefore, early diagnosis of cancer is vital to patient survival.

Advanced and mid-stage disease typically cause patient discomfort leading to physician visits and diagnosis of cancer via visualization with CT, MRI, or ultrasound imaging followed by physical biopsy. In contrast, early stage cancer is usually asymptomatic and escapes detection until after the optimal treatment window has passed. For many years, sensitive and noninvasive techniques have been sought to enable early detection and effective diagnosis, which will greatly improve patient prognosis. The effectiveness of a diagnosis is commonly reported in terms of sensitivity and specificity. Sensitivity is the percentage of the total diseased (positive) specimens or persons tested which are identified as positive by the
test. Similarly, specificity is the percentage of the total disease-free (negative) specimens or persons tested which are identified as negative.

\[
\text{Sensitivity} = \frac{N_{\text{True Positive}}}{N_{\text{True Positive}} + N_{\text{False Negative}}} \tag{1}
\]

\[
\text{Specificity} = \frac{N_{\text{True Negative}}}{N_{\text{True Negative}} + N_{\text{False Positive}}} \tag{2}
\]

A useful diagnostic generally needs to perform with both high sensitivity and specificity.

1.2 Optical Techniques for Cancer Detection

Techniques involving optical imaging and optical spectroscopy have been shown to capture relevant diagnostic information including cellular metabolic activity\(^2\), extracellular structural information\(^3\), hemoglobin and oxygenation levels\(^4\), and pH\(^5\). Optical imaging in tissues is limited by light penetration depth, but optical techniques remain extensively applicable because early cancer very frequently arises at the epithelial (outermost) organ surface. Optical techniques have proven feasible for cancer detection in several noninvasively accessible organs such as skin\(^6\), oral cavity\(^7\), cervix\(^8\), and gastrointestinal tract\(^9\).

Light based techniques can be categorized according to their measurement of scattered light (elastic or inelastic), or fluorescence. Elastic scattering (more commonly “reflectance”) describes the case in which a photon interacts with a particle (but is not absorbed) and is redirected maintaining its original energy and wavelength. Inelastic scattering (Raman scattering) is a similar but much weaker effect that includes a change in energy and wavelength shift of the photon. This body of work is concerned most with the third phenomenon, fluorescence, wherein the photon is absorbed and then quickly re-emitted with less energy (longer wavelength). In fluorescence, incident photons lose energy to
vibrational relaxation (Fig. 1) and the resulting spectral (“Stokes”) shift allows their separation from scattered photons using an optical filter or dispersive element.

Fluorescence is produced only by molecules of an appropriate chemical structure called fluorophores.

1.3 Autofluorescence and Optical Instrumentation

Autofluorescence (AF) refers to fluorescence from endogenous fluorophores, which exist naturally in all tissue. These include the metabolic cofactors reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), the structural extracellular component collagen, and amino acid tryptophan. See Table 1 for optical properties of endogenous fluorophores\(^\text{10}\). AF imaging is a potentially useful diagnostic technique because no preparation of the tissue is required and the fluorescence intensities reflect concentrations of endogenous fluorophores shown to change with disease progression.
Table 1. Optical properties of endogenous fluorophores

<table>
<thead>
<tr>
<th>Endogenous Fluorophore</th>
<th>Excitation Maxima (nm)</th>
<th>Emission Maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>280</td>
<td>350</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>275</td>
<td>300</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>260</td>
<td>280</td>
</tr>
<tr>
<td><strong>Structural Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>325, 360</td>
<td>400, 405</td>
</tr>
<tr>
<td>Elastin</td>
<td>290, 325</td>
<td>340, 400</td>
</tr>
<tr>
<td><strong>Metabolic Coenzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>450</td>
<td>535</td>
</tr>
<tr>
<td>NADH</td>
<td>290, 351</td>
<td>440, 460</td>
</tr>
</tbody>
</table>

From a fluorescence instrumentation standpoint, all systems require illumination light source (excitation side), detection system (emission side), and a means of strongly excluding the illumination wavelengths from detection. Fluorescence intensity is very weak compared to reflected light intensity and requires high suppression of the reflected light (optical density of five or more). Fiber optic probes and spectrophotometers may be used to capture fluorescent emission data with high spectral resolution. However, diagnostic use in tissue is limited by the time required to capture a large number of measurements from different spatial positions. Spectral imaging (also multispectral or hyperspectral), which consists of acquiring several uniquely filtered images in succession, lends itself more easily to tissue diagnostics with the ability to quickly interrogate much larger areas but, in general, sacrifices spectral resolution. Snapshot spectral imagers attempt to simultaneously capture images and full spectral information.

AF for cancer diagnosis goes back to the 1980’s when studies with laser induced fluorescence spectroscopy (LIF) of tissue commenced. Detailed summaries of the studies and findings exist in the literature^{10-12}. Multi- and hyper- spectral imaging of tissue have
matured as well since this time with several systems being commercialized\textsuperscript{13}. Even so, certain areas of AF diagnostics are only now being explored. These include use of new excitation and emission wavelengths\textsuperscript{14}, time resolved AF measurements\textsuperscript{15}, multiphoton excitation\textsuperscript{16}, and heavy emphasis on in vivo measurements.

1.4 Research Objective #1: Diagnostic Autofluorescence Imaging of the Ovary

Three related publications by the dissertation author are presented and discussed. The first of these is an ex vivo AF imaging study of normal and cancerous human ovaries from thirty patients\textsuperscript{17}. The feasibility of diagnostic AF imaging of human ovary was indicated by previous AF spectroscopy measurements of the organ\textsuperscript{18}. The present study was conducted with a multispectral imaging system (365 nm excitation and 8 fluorescence emission images collectively spanning the spectral range 400 to 640 nm) provided by Apogen Technologies (now QinetiQ North America). Freshly resected ovaries were imaged to investigate the value of this technology for use in laparoscopic screening of high risk patients. Current practice is removal of the ovaries of women deemed high risk for ovarian cancer as a preventative measure.

AF imaging has been used to diagnose cancer in the human cervix\textsuperscript{19}, colon\textsuperscript{20}, and oral cavity\textsuperscript{7}, using similar wavelengths but no such study of this magnitude had existed for the human ovary. Diagnosis of the ovarian AF images was also performed in a novel manner. Specifically, the images of whole human ovaries were evaluated pixel by pixel with classification as normal or cancer based on comparison of the spectral data to a library of previous single point spectroscopic measurements of ovarian biopsies. Comparison of spectral data originating from an imaging system to data from a non-imaging system is useful
as it may strengthen diagnostic accuracy by making use of a more extensive set of spectral data and corresponding histopathology.

1.5 Research Objective #2: Deep UV Diagnostic Autofluorescence Imaging

The second publication of the dissertation describes the development of a novel multispectral imaging system and its application to diagnosis of human colon cancer surgical specimens. In colon screening, techniques are sought to reduce the polyp miss rate, which is significant for small and/or flat lesions. Currently no single technique has supplanted white light video endoscopy as the standard screening technique, but several enhancements to white light endoscopy are being investigated yielding a multimodal approach. Techniques include chromoendoscopy (CE), magnification enhancements, narrow band reflectance imaging (NBI), and autofluorescence (AF). In CE a dye such as indigo carmine is administered to the colon to increase lesion contrast. This method has shown increased detection ability for dangerous flat lesions but has not been well adopted because of additional time requirements. Optical magnification and confocal endomicroscopy have been used to successfully reveal minute lesion structure and predict histology of small polyps. NBI is a technique involving capture of multiple narrow band reflectance images, which are combined in a pseudocolor image to enhance polyp visualization. Magnification or NBI features are included in several newer model colonoscopes.

Autofluorescence imaging for colon screening has been implemented commercially. Olympus manufactures a dual AF and white light reflectance colonoscope called the AFI system. The AF mode illuminates with blue light (395-475 nm) to excite fluorescence images and with green light (540-560 nm) for reflectance reference image. These two images are combined presenting a pseudocolor image to the physician. A second AF colonoscope
under development, the PINPOINT system from Novadaq evolved directly from LIFE-GI\textsuperscript{26}, the first AF colonoscope to approach commercialization. PINPOINT is quite similar to AFI, illuminating with blue light (400-450 nm) for fluorescence and red light (>630 nm) for reflectance images and combining the two images to produce a pseudocolor image.

The multispectral imaging system produced for the current study seeks improvement via use of short UV excitation wavelengths and novel ratio images. The commercial AF scopes described have not proven a significant detection advantage over established white light video endoscopy\textsuperscript{27-30}. The spectral imaging system developed utilizes excitation wavelengths from 260 nm to 650 nm allowing study of multiple fluorophores. Extending the available excitation wavelengths deep in the UV (below 300 nm) allows UV imaging of the fluorescent amino acid tryptophan and proteins. Prior fluorescence spectroscopy in humans has shown cells from colon cancer displaying two times greater autofluorescence in response to 280 nm excitation than normal cells of the colon\textsuperscript{31}. Development of a unique illumination system (full spectrum xenon arc lamp with UV cold mirror, quartz coupling optics, and quartz fiber bundle) was key to the success of this study. Nonstandard elements were needed because lenses and optical fibers of standard material cannot accommodate light below 330 nm due to heavy absorption.

The techniques used for enhanced visualization of cancerous and precancerous lesions were based on spectral analysis of fluorescence images targeting tryptophan, collagen, NADH, and FAD. Narrowband diffuse reflectance images were also studied and incorporated. Ratio images combining various image types were investigated during the study for use in differentiating difficult to visualize lesions from normal colon tissue.
1.6 Research Objective #3: Deep UV Diagnostic Autofluorescence Microscopy

The third publication of this dissertation focuses on a novel AF microscope and its application to diagnostic imaging of pancreatic cells. Fluorescence microscopy is a technique that has been widely applied to study cell structure/organization as well as molecular pathways involved in disease progression. Because exogenous fluorophores can be highly specific and provide labeling intensities that are orders of magnitude higher than AF signals, one often introduces a fluorescent label with affinity for a particular cell structure. The alternate approach (and the one utilized in this study) is to develop equipment sensitive enough to capture a high quality image of very weak AF. Label free microscopy is ideal for diagnosis where it is desirable to interrogate the biological system without disruption of its current processes and physiologic state. The fluorophores probed in AF microscopy of cells are the same as those encountered in macroscopic tissue imaging (refer to Table 1) with the exception that little if any connective tissue with collagen or elastin is present.

The microscope developed for the study is unique in its ability to excite tryptophan autofluorescence with deep UV light. It utilizes the same illumination system developed for the macroscopic AF imaging system used to image the colon and provides light from 260 to 650 nm. The cell type chosen for study was pancreatic, as no published tryptophan AF images exist for this organ, and the ability to diagnose these cells is directly applicable to evaluation of pancreatic needle biopsy. Sub-300 nm wavelengths allow visualization of protein content in cells due to fluorescence of the amino acid tryptophan. The ability to make optical measurements of protein levels is of great interest in cancer research because
they are tied to cellular growth processes affected by cancer. The current study seeks to understand the diagnostic value of cellular tryptophan AF.

Research in microscopic imaging of cellular tryptophan AF is a new area of investigation limited predominantly to papers published in the past six years. Microscope techniques used in past studies have included both single photon and two photon excitation. Studies have included imaging of human fibrosarcoma cells, human foreskin keratinocytes (HFK), cervical cell lines, and esophageal biopsies. Findings have focused primarily on the unique visualization of bright nucleolus and relatively diffuse cytoplasmic AF. One author reported increased tryptophan AF intensity in immortalized HFK’s compared to primary HFK’s. The current study expands this effort to the pancreas, characterizes the signals observed, and attempts to distinguish normal and cancerous cells as proof of principle for a cellular diagnostic test.
CHAPTER 2: PRESENT STUDY

Details of the dissertation research are presented in the published papers included as Appendix A, B, and C. This chapter summarizes findings related to each appended publication.

2.1 Autofluorescence Imaging of the Human Ovary

The great potential of spectral imaging is that spectroscopic information, proven to have diagnostic value in numerous organs, can be collected not only at a single point location but also simultaneously over many different spatial locations to screen for diseased areas. Successful techniques will have application in disease screening, surgical tumor excision guidance, and guidance of tissue biopsy. The challenge of AF imaging lies in identifying the most diagnostically relevant information out of a very large pool. This study relies on well understood mathematical techniques for accomplishing this task.

2.1.1 Summary of Methods

A pre-existing multispectral imaging system and predefined imaging sequence were used to image whole human ovaries following excision from consented oophorectomy patients. Histopathology reports for the specimens were later obtained and used as gold standard diagnostic information.

For each ovary imaging sequence, the collected data included 16 uniquely filtered images (8 AF and 8 reflectance). For AF, excitation was at 365 nm, and eight different emission filters were centered at 420, 440, 455, 485, 510, 530, 560, and 600 nm. Reflectance images were illuminated with linearly polarized white light and collected in one of two polarization configurations. Parallel polarization produced standard images in blue, green,
red, and near infrared (NIR) bands, while perpendicular polarization produced reduced-glare images in blue, green, red, and NIR.

The goal was to discriminate between cancerous and normal ovaries based on the AF images and reflectance images. Prior to analysis, image data were calibrated to account for day to day fluctuations in the light source intensity. This allowed investigation of observed differences in absolute AF intensity. Because AF images at 365 nm excitation excite primarily collagen and NADH, they represent a combination of structural and metabolic tissue characteristics. Techniques exist, such as parallel factor analysis,\textsuperscript{18} that can separate the overlapping AF emissions of two or more fluorophores but these require additional spectral information (multiple excitation wavelengths). For this reason, the analysis utilized focuses on trends in the data that can be exploited for discrimination with little emphasis on the functional origin of those differences.

Because the surface of even a normal ovary can be very heterogeneous (creases, pits, microvasculature), it was informative to analyze and classify each pixel of each ovary image. Each pixel is assigned a given diagnosis based on its AF data’s correspondence with AF data from tissue of known diagnosis. However, histopathology information, which is taken as truth for classification, was limited in this study to a single gross diagnosis for each whole ovary. Therefore, it was useful to utilize a large collection of histopathology-proven AF data from a separate previous study to train classification. The training data consisted of AF measurements on human ovarian biopsies taken with a fiber optic probe and required conversion to match the spectral data acquired in the imaging study. High spectral resolution, multiple excitation measurement was reduced to only 8 AF intensity values and scaled to match the current study as described in Appendix A.
The mathematical method called principal component analysis (PCA) was used to analyze the variability of the data and determine how it might be used most efficiently for classification. PCA takes the input variables (8 unique AF intensity values) and transforms them to a new set of variables (principal components, PC), which are uncorrelated. The typical result is that the variability of the data is described adequately by a smaller number of variables. The highest scoring PC’s reveal distinguishing trends in the data.

A second mathematical method called linear discriminant analysis (LDA) was used to classify the data after its transformation by PCA. LDA requires a training set, or bank of measurements including their correct assignments to one of the possible classes (cancerous ovary or normal ovary). For each set of measurements of unknown class, LDA determines the most probable class membership based on the training measurements. In this study LDA is applied to each pixel of the image in turn assigning a diagnosis.

2.1.2 Summary & Discussion of Results

The study was unique not only for being the first of its nature in the human ovary but also for its utilization of spectral data from two different instruments. Spectral data obtained from normal ovary using the AF imager and the fiber optic probe were compared prior to data analysis (Figure 6, Appendix A). The average shapes of eight point spectra acquired by the two instruments from presumably equivalent tissue differed greatly despite each instrument undergoing spectral calibration. Emission spectra collected by the imager were red shifted compared to those from the fiber optic probe. This observation was surprising until one considers the differences in excitation-emission geometry of the two systems. The imager with a large working distance of 30 cm illuminates a circular area larger than the 4 cm x 3.3 cm field of view (and very much larger than a single pixel), while the fiber optic
probe is a contact device illuminating and collecting from the same 0.8 mm circular region. It may be that red photons are selectively collected in the imaging configuration because the AF photons collected traverse longer paths within the tissue (on average), and absorption is reduced in the red region. A similar observation was reported by Gebhart.\textsuperscript{36} Therefore, one key result of this study was the determination that shape correction of AF spectra will be necessary for direct comparison of spectra from an imaging system and a single point probe measurement.

Inspection of the average spectra from both systems confirmed previous observations of typically lower AF (at 365 nm excitation and all emission wavelengths) from cancerous surface tissue compared with normal surface tissue. Multiple factors can contribute to this result. Reduction in collagen AF is a likely factor, presumably due to growth, which disrupts the collagen network\textsuperscript{3} and destroys highly fluorescent crosslinks. NADH is believed increased in cancer but overshadowed by the signal from collagen. Collagen and NADH contributions were not investigated further in this study. Increases in absorption by blood component hemoglobin can also contribute as will be discussed later.

Although reduced AF emission is common in cancerous ovaries, the study showed that this alone is not sufficient for effective diagnosis. Consider the principal component analysis of the training data. The highest scoring principal component closely resembles the average spectrum (Figure 8, Appendix A) indicating that intensity is a good starting point for identifying cancer. Ultimately what matters in optical diagnosis is that high sensitivity and specificity can be achieved. Diagnosing ovaries based on AF intensity (single principal component) was effective in identifying diseased ovaries (100% sensitive); however this
technique produced too many false positives (38% specificity, Table 2, Appendix A). The use of a second principal component lead to increased specificity of 51.1%.

Classification maps, or diagnostic maps, of ovaries imaged were produced and found to be very useful in visualizing the heterogeneity of the pixel spectra collected (Figure 10, Appendix A). It became clear upon inspection of these maps that areas of ovaries higher in vasculature and hemoglobin content were being misclassified as diseased. We investigated the possibility of correcting for the effect of varying hemoglobin across an image by dividing AF images by crossed polarization reflectance image. This works in principle because AF images and reflectance images are affected by hemoglobin absorption in the same way, and the effect is partially canceled by considering a ratio of AF to reflectance. It was unknown what reflectance band would produce the best correction, so each band (Blue, Green, Red, and NIR) was tested. The ratio of AF to green reflectance produced the greatest improvement in classification. The specificity of ovary diagnosis increased from 51.1% using AF data and two PC’s to 68.5% when using a ratio of AF to measured green reflectance.

Before sensitivity and specificity could be calculated for the imaging method, criteria needed to be established. It was chosen that when >90% of the ovary image region of interest (ROI) classified as normal, the overall ovary classification would be considered normal. When <90% of the ovary image ROI classified as normal, the overall classification would be considered cancer. These criteria successfully produced sensitivity and specificity approaching that predicted by a jackknife analysis of the training data set. Inherent to this assessment of the diagnostic value of imaging was a supposition that the gross
histopathology assessment, which samples only a small percentage of each ovary, never failed to detect disease through sampling error or otherwise.

If the imaging technique is to be trusted for surgical guidance, it would be important to perform more extensive histopathology coordinated with imaging. This would confirm the reasons (or lack of reason) for apparent misclassification of specific tissue areas. Histopathology was limited in this study by demands on the time of the pathologist.

The sensitivity of the technique in this study may be unrealistically high (100%) due to the small number of cancerous ovaries acquired for the study, however the training set which contained a larger number of cancerous samples also produced 100% sensitivity. The specificity at approximately 70% was lower than would be desired. Effectively, three of every ten normal ovaries falsely tested positive for cancer. In this case the limitation may have been the single excitation wavelength of the instrument. Several spectroscopic studies have shown improvement in diagnostic effectiveness by using two or more excitation wavelengths, but this adds to the cost and complexity of the instrument.

2.2 Autofluorescence Imaging of the Human Colon

While the AF imaging study of human ovarian cancer provided useful results, the instrument did not allow for images to be collected with multiple excitation wavelengths. Prior to the colon cancer study a new AF imaging system was developed in our laboratory capable of ten different excitation wavelengths including output as low as 260 nm. AF imaging studies at sub-300 nm excitation wavelengths are rare because of specialized system requirements. For example, the most common optical glass N-BK7 is unsuitable, as it does not transmit light well below 330 nm. Sub-300 nm excitation provides additional diagnostic information by inducing AF from the amino acid tryptophan and the proteins containing it.
Colon cancer was selected for study because of the reported need for a technique that can reliably detect flat lesions. These growths in the wall of the colon are neither raised nor discolored, making them very difficult to detect with a standard video endoscope, yet they often develop into cancer if left untreated. The colon imaging study was undertaken with a goal of determining specific spectral imaging techniques that make flat and difficult to see lesions appear highly visible. Ultimately, a practical AF imaging system for the colon requires an endoscope, but none is needed at this stage of technique development due to availability of excised tissue.

2.2.1 Summary of Methods

The prototype AF imaging system used in the colon imaging study (Figure 2) was developed in our laboratory through my efforts as well as earlier lab member contributions. (See also a system diagram as Figure 1 of Appendix B)
The camera, UV objective lens, emission filter wheel, mobile arm, and quartz fiber optic bundle were purchased, assembled, and tested prior to my joining the research lab. I integrated these components in a new mobile platform (wheeled black cabinet) together with
a new computer, xenon arc lamp system with built-in filter wheel, and filter wheel controller. A custom LabView interface and scripting language were developed previously in our laboratory and used for instrument control. I developed the script command sequences, automating collection of desired spectral images by repeatedly specifying open and close of lamp shutter, filter wheel changes, image acquisitions, and storage of data.

Development and characterization of the illumination system was a major task and crucial to the success of the AF imaging system. A single source (xenon arc lamp) was chosen to simplify optical design by providing sufficient optical output at all desired illumination wavelengths. However, the use of a broadband source also necessitated the extensive use of optical filters for excitation wavelength selection. Optimal illumination bands for AF imaging were identified previously by earlier graduate students in the lab based on known fluorophores and measurements of excitation-emission matrices of tissue. These excitation bands were centered at 280, 320, 340, 370, 400, 415, 440, 480, and 555 nm. Appendix D of this manuscript documents specific excitation and emission filters used in the imaging system and presents the results of filter performance testing.

In order to perform the imaging study, surgical specimens of colon were obtained from consenting patients undergoing colectomy for removal of cancer or polyps. Intact sections of colon containing one or more lesions were imaged typically within one hour of resection. Cylindrical specimens were sliced open lengthwise, rinsed thoroughly with saline, and positioned carefully beneath the camera for imaging. It was important to frame the images so that both normal colon and lesion can be seen in a single image representing a 4 cm by 4 cm area at the specimen. By demonstrating lesion contrast in a single image, we
could simulate what a physician might see using an advanced endoscope of the same technology.

Because it was unknown which AF images would prove to be most useful, a large number of uniquely filtered images were captured in succession. Six different excitation bands were chosen (280, 320, 340, 370, 400, and 440 nm) and as many as four different longpass filtered emission bands per excitation band. Crossed polarization reflected light images were also collected at 370, 400, 415, 440, 480, and 555 nm excitation by utilizing no emission filter. In total, each image set consisted of 27 uniquely filtered images and 27 corresponding dark measurement images. For the AF images, long exposures of one or two seconds were needed resulting in an imaging sequence spanning about 90 seconds.

Initial evaluation of cancer images was visual with the criterion for success being how clearly one could identify lesions within the image. Given the very large number of uniquely filtered AF images, attention was devoted to six images (F280, F320, F340, F370, F400, and F440) targeted to specific tissue fluorophores. These image types were named according to excitation wavelength, and their emission bands are documented in Table 1 of Appendix B. A quantitative measurement was desired for image evaluation and established through selection of a contrast metric. Weber contrast (Equation 3) was the initial evaluation metric. In order to utilize the metric, regions of interest (ROI) were manually defined identifying the ‘Lesion’ and ‘Normal’ pixel regions of each specimen image. The calculation is performed using the mean pixel intensities in the respective ROI’s.

\[
C_{\text{Weber}} = \frac{I_{\text{Lesion}} - I_{\text{Normal}}}{I_{\text{Normal}}} \tag{3}
\]

Ratio images combining two or more image types were pursued as a means to enhance the useful contrast and achieve even more effective lesion visualization. Table 2
presents the formulas for thirty ratio images that were the subject of study. A modified version of the contrast metric (Equation 4) was used to evaluate ratio images.

\[ C_{opt} = \frac{|I_{Lesion,75\%} - I_{Normal,75\%}|}{\sigma_{LN}} \]  

(4)

In this contrast metric, 75\textsuperscript{th} percentile pixel values of the respective ROI’s are used in place of the mean, and normalization is by the pooled standard deviation of the respective ROI’s.

| \( R1 \) | \( R11 \) | \( R21 \) |
| \( \frac{F280 * (F440 + F320)}{F280 + F320} \) | \( \frac{F280}{F440} \) | \( \frac{F440}{R555} \) |
| \( R2 \) | \( R12 \) | \( R22 \) |
| \( \frac{F440 + F320}{F280} \) | \( \frac{F440}{F320} \) | \( \frac{F440}{F440} \) |
| \( R3 \) | \( R13 \) | \( R23 \) |
| \( \frac{F440 + F320}{F280 * F340} \) | \( \frac{R555}{1} \) | \( \frac{1}{F440red + F440} \) |
| \( R4 \) | \( R14 \) | \( R24 \) |
| \( \frac{F440 * F320}{F280} \) | \( \frac{R400}{F340} \) | \( \frac{1}{R555 + F440} \) |
| \( R5 \) | \( R15 \) | \( R25 \) |
| \( \frac{F440 * F320}{1} \) | \( \frac{F340 + F440}{R440} \) | \( \frac{R555 * F440}{1} \) |
| \( R6 \) | \( R16 \) | \( R26 \) |
| \( \frac{F440 + F320}{1} \) | \( \frac{R415}{R480} \) | \( \frac{(F440)^2}{F340} \) |
| \( R7 \) | \( R17 \) | \( R27 \) |
| \( \frac{F440 * F320}{F340} \) | \( \frac{1}{R440} \) | \( \frac{F340}{F440} \) |
| \( R8 \) | \( R18 \) | \( R28 \) |
| \( \frac{F440 + F320}{F340} \) | \( \frac{R440 * R400}{R440} \) | \( \frac{F440 + F340uv}{F320blue} \) |
| \( R9 \) | \( R19 \) | \( R29 \) |
| \( \frac{F440 * F320}{1} \) | \( \frac{R370 * R480}{R440} \) | \( \frac{F320}{R480} \) |
| \( R10 \) | \( R20 \) | \( R30 \) |
| \( \frac{1}{F440} \) | \( \frac{1}{R555} \) | \( \frac{1}{R555} \) |

Table 2. Formulas for ratio images investigated. ‘F’ prefix designates fluorescence image. ‘R’ designates reflectance image. Three digit number indicates image illumination wavelength. Emission bands are described in Table 1 of Appendix B.

Techniques were sought to display ratio images most effectively for visual inspection. These included a saturation scaling method whereby the display range is set to saturate a small percentage of image pixels at both the high and low ends of intensity histogram. A
second method was histogram equalization, which scales the pixel intensities nonlinearly to produce the desired flat histogram shape.

Successful ratio images were ultimately compared to ratio images simulating those of commercial AF endoscope systems for the colon. The spectral imaging bands used were well matched to those of Olympus’ AFI system and Xillix Technologies’ LIFE-GI system.

2.2.2 Summary & Discussion of Results

The first observation of the imaging study was that colonic lesions consistently had lower image intensity compared with the surrounding normal tissue. In fact, on average, darker lesions were observed in all uniquely filtered AF and reflectance images. One of the key hypotheses and motivations for this study was a prediction that sub-300 nm excitation would produce higher AF intensity in lesions due to increased tryptophan and protein content. A prior study indicated that cells obtained from colonic adenocarcinoma produced twice the tryptophan AF of cells obtained from surrounding normal colon. One explanation for the conflicting observation in the current study could be the increased presence of blood vessels and the absorber hemoglobin in diseased tissue which would have been absent in both isolated cell samples. This explanation is supported by the reddened appearance of many lesions. Also the lesion contrast data of the reflectance images follows a trend matching the hemoglobin absorption curve (Figure 3, Appendix B).

A second clear trend identified in the data was that AF images excited at 440 nm (F440) produced better average lesion contrast than any other AF or reflectance image collected (Figures 3, 4, Appendix B). This result agrees with previous findings that blue excited AF is useful for identifying growths in the colon. In fact, this is the basis for contrast in two current AF colonoscopes. Because of a large variation in the data, the
increased F440 lesion contrast was, however, not statistically significant compared to many of the other image types.

Notably, the colonic lesions imaged were at various stages of disease progression from small polyps (benign) to large tumors encircling the colon. As one might expect, the advanced lesions (adenocarcinomas) resulted in greatest image contrast. The precursor lesions (adenomas), which were generally more difficult to see with the naked eye, produced less contrast in the spectral images as well. In order to determine which AF or reflectance image provides the greatest advantage, the average lesion contrast was quantified from a subset of lesions that were difficult to visualize in standard color images. In the so called ‘low contrast lesions’ or LCL’s, AF images excited at 440 nm demonstrated a lesion contrast advantage over all others which was significant under a T-test at the p<0.05 level.

In an effort to improve on the AF and reflectance images, ratio images were composed. Goals of ratio image formulation were to increase lesion contrast, to make lesions appear brighter than normal tissue, to correct for the influence of varying hemoglobin distributions, and to correct for nonuniformity of illumination.

The primary attempt to correct for hemoglobin absorption was $R_{13} = \frac{F_{280}}{R_{555}}$. Previous work suggested that division by a green reflectance image could be an effective correction. Resulting $R_{13}$ images displayed increased intensity from the lesion portion of images suggesting the reflectance image may have compensated well. Although the “corrected” tryptophan fluorescence image ($R_{13}$) provided positive lesion contrast, it proved to not be as effective as other ratio images at highlighting diseased areas. Evaluation of a large number of images showed that the calculated lesion contrast in a ratio image (Equation 4) needs to exceed a value of approximately 3 for a lesion to be highly visible. $R_{13}$ failed to
meet this criteria for any of the LCL’s and was not considered one of the most valuable ratio images for diagnosis.

General conclusions on ratio imaging were drawn based on inspection of the many ratio images presented in Table 2. Reversing the contrast (to produce bright lesion and dark normal tissue) was performed in any image by taking the reciprocal (e.g. R10, R14, R20). Correcting for nonuniform illumination was best accomplished with a simple traditional ratio image of the form A/B. More complex formulas, especially those involving multiplication, produced high contrast but the very large spread of ratio image intensity values made it difficult to display them without significant saturation and loss of visual detail. It was these images that prompted the investigation of the optimized contrast metric (Equation 4), which accounts for the negative effects of large intensity standard deviation. Testing of the new metric showed a high correlation with visual assessment of contrast.

Through a process of comparing calculated contrast and grouping visually similar ratio images the number of ratio images of interest was reduced from thirty to the best nine (Table 3, Appendix B). Deep UV excitation does not appear necessary as image F280 appears in none of the selected ratio images. Independent visual evaluation of nine ratio images by seven investigators was used to identify the most important ratio images. Each image was rated inadequate, adequate, or exceptional in terms of ease with which the lesion could be identified. While R10 (= 1 / F440) produced the best results in lesions most visible in white light (non-LCL’s), two new ratios stood out for performance in viewing LCL’s. R27 (= F340 / F440) and R30 (= R480 / R555) produced more exceptional images of LCL’s than any other ratio. 50% of LCL’s were imaged exceptionally by R27 and 37.5% by R30. Perhaps more interesting was the discovery that nine of ten LCL’s could be imaged
exceptionally by either R27 or R30. This indicates that the two ratio images are complementary in nature.

The discovery of ratio images R27 and R30 indicates that both AF and narrow band reflected light images are valuable in screening the colon, but their simultaneous use (such as through a split screen) would produce maximum contrast in the greatest number of lesions. R27 demonstrated the usefulness of adding a UV excitation wavelength to AF colonoscopes.

To establish the value of these findings, they must be compared to current instrumentation. The AFI colonoscope from Olympus provides lesion contrast based on differences in a ratio of reflected green light to blue-excited AF emission. Because an AFI system was not available for the study, the ratio image R22 was constructed to simulate images from Olympus AFI. A second AF colonoscope produced by Xillix Technologies was simulated with a ratio image R21. The desired ratio images of all LCL’s were generated using the previously collected data. Both visual analysis and quantitative contrast analysis showed an advantage for R27 versus R22. 80% of R27 LCL images were visually adequate versus only 50% of R22 LCL images, and the mean contrast metric score was 51% higher for R27, although significance of the increase was p=0.24. Therefore, more data are needed to definitely establish the improvement of new ratio images, but a trend toward more effective colon imaging technique is established. Indications are that a system combining ratio images R27 and R30 will further enhance the ability to survey the colon for lesions. The most straightforward method of utilizing both ratio images is through a split screen display monitor.

The findings of the AF imaging study of the colon point the way for an advanced colonoscope, which is the subject of future work for the laboratory. One of the ratios
(R30=R480/R555) could be achieved through narrowband reflectance imaging, a technique currently available in several modern colonoscopes. The most successful ratio image (R27=F340/F440) requires changes in hardware allowing ultraviolet illumination. In order for ultraviolet illumination to be routinely used in humans, the safety threshold for UV exposure in the colon must be proven. Mouse models are the likely avenue for this task.

2.3 Deep UV Autofluorescence Microscopy of Pancreatic Cells

In the colon imaging study, sub-300 nm light was used to view tryptophan AF macroscopically over a 4 cm x 4 cm specimen field of view. In those images each pixel represented an area 80 um x 80 um, and integrated the AF from a large number of cells. Replacing the optics with a microscope objective and using the same illumination allows resolution of tryptophan AF from individual cells and subcellular features. The AF image provides unique visualization of proteins and may provide diagnostic information, as enhanced protein production is likely needed to support cancer growth.

In order to diagnose cancer, especially in the pancreas and breast, masses are often sampled using a needle biopsy to collect cells. Evaluation of the cells requires sample transportation, a staining process (such as a Papanicolaou stain), expert evaluation by a cytopathologist, often review by other cytopathologists, and generation of a report. The process is expensive and slow. AF microscopy of tryptophan may provide an objective, label-free diagnostic that could be performed quickly by a non-expert following needle aspiration.

2.3.1 Summary of Methods

AF microscopy of cells was performed using the same ultraviolet/visible illumination system described in 2.2.1 and Appendix B. Excitation wavelengths of 280 nm, 370 nm, and
440 nm and bandpass emission filters centered at 360 nm, 447 nm, and 560 nm were used to collect AF of tryptophan, NADH, and FAD respectively. The microscope was an upright model from Olympus with its construction allowing emission filters to be manually inserted above the objective. Cells were illuminated from below the stage in a transillumination configuration. The 40X objective was designed for UV imaging at wavelengths as low as 340 nm and provided submicron resolution.

Two cultured pancreatic cell lines were chosen to investigate the distribution of short wavelength AF and its subcellular origin. These were the MIA PaCa-2 cancer cell line and hTERT-HPNE immortalized ductal cell line. For imaging, each cell line was cultured on glass coverslips in a complete growth medium at 37º C with 5% CO₂. Imaging occurred on a heated stage with coverslip inverted over a shallow well of low AF imaging media on a quartz slide (See Figure 2 of Appendix C). Organelle-specific fluorescent labels including probes for mitochondria, lysosomes, nuclei, and endoplasmic reticulum were tested.

In preparation for work with human samples, primary cells were acquired from rat pancreas, and a protocol was developed for dissociating tissue into individual cells and small clusters (See Appendix F). In short, the tissue was minced with a blade and incubated with collagenase to digest the extracellular matrix. Primary pancreatic cells for imaging were later harvested from humans using this optimized dissociation protocol.

To test the hypothesis that 280 nm excited AF image intensity indicates protein levels, an experiment was devised which chemically modulates cellular protein. Cells were treated by a proteasome inhibitor (Velcade), which indirectly causes protein levels to increase by stopping their normal degradation as synthesis continues undisturbed. Treated
and untreated cells were imaged on coverslips and their AF intensities compared at various time points.

Images were processed prior to intensity analysis by first subtracting a dark image (light source shutter closed). AF background was then removed by subtracting an AF image of a coverslip containing no cells. A flat field correction was performed to correct for reduced intensity observed near the edges of the image field. A fluorescent tape (uniform object) was imaged for this purpose and correction applied through division by a normalized smoothed version of this image.

The AF of imaged cells was quantified as AF intensity per pixel. After investigation of several tryptophan AF images of cells, a threshold value was set and used to select pixels corresponding to cellular material. The number of pixels selected was noted. The AF intensity from selected pixels was summed and then divided by the total selected pixels. This resulted in a single measurement from each image representing average cell AF intensity.

2.3.2 Summary & Discussion of Results

AF images of cultured cells excited at 280 nm were distinct from AF images of the same cells excited at 370 and 440 nm. Specifically, the short wavelength images (attributed to tryptophan) were observed to display bright AF over the entire extent of and to the far reaches of the cytoplasm. In general, the distribution of intensity was more diffuse than at longer excitation wavelengths. However, in some cells a bright nucleolus was easily identified within the nucleus, and bright unidentified punctate regions were sometimes seen in the cytoplasm. Nuclei were darker than cytoplasm but still produced significant intensity.

When a proteasome inhibitor was administered to cultured cells (allowing protein levels to rise by inhibiting natural breakdown processes), clear increases in measured
tryptophan AF intensity were observed. Cellular AF intensity per pixel increased progressively with treatment time reaching a level 40% higher than control cells within 36 hours. A protein synthesis inhibitor administered together with the proteasome inhibitor was found to partially block the increase in AF, reducing the observed increase by one half. Together these results provide evidence that 280 nm excited AF images can be used to measure cellular protein levels. The 40% change in AF presumably approximates the maximum variation in the tryptophan signal for a given cell line.

Fluorescent probes designed to target endoplasmic reticulum (ER), mitochondria, lysosomes and nuclei were investigated to assess the origin of AF in cultured cells. Comparison of resulting images to 280 nm excited AF images indicated that none of these organelles is dominant in its contribution to the AF image. Similarity was seen between the AF images and both the ER and mitochondrial probe images. The ER images showed signal extending to the far reaches of the cells. The brightest regions of cells in mitochondrial probe images corresponded well to brightest regions of cells in the AF image. The AF images were more diffuse than any of the probe images and revealed much less structure in the cytosol. This indicates that tryptophan and proteins are ubiquitous in the cell producing a relatively uniform AF signal.

To simulate cells obtained from needle biopsy, pancreas tissue was obtained from rat and dissociated to release individual cells. The suspended, rounded up, secretory cells obtained were imaged for comparison to the cultured cells. The appearance of suspended secretory cells in 280 nm excited AF images was vastly different from the non-secretory cultured cells. Areas of bright AF were observed at one side of cells while the opposite side was darker. For small clusters of cells, very bright AF was observed toward the center of the
clusters. Nuclei were difficult to observe in these tryptophan images. The well defined AF pattern observed was identified as AF of secretory granules. Pancreatic secretory cells produce digestive enzyme precursors and secrete them into ducts as needed for digestion. Staining confirmed the protein-packed granules as the likely source of the bright AF signal.

The same dissociation technique was applied to human pancreas tissue allowing image capture of human secretory cells. The resulting tryptophan images of primary cells were very similar to observations in rat; however, the areas of bright AF were not as restricted to a single side of the cell. Thus, the polarity in the AF pattern was reduced, and cell nuclei were also more easily identified as dark circles.

An additional experiment, undocumented in the journal paper prepared for publication (Appendix C), was the imaging of primary human pancreatic exocrine cells after short-term culture. Large clusters of dissociated secretory cells were deposited on coverslips and cultured in complete media for 48 hours. Cells adhered to the coverslip and spread out to form a monolayer. Sample AF images of these cells are presented in Figure 3. The first image (tryptophan AF) shows very clearly visible dark nuclei and some patches of brighter AF that may indicate secretory granules. The texture of cytoplasmic AF is similar to that observed in images of suspended primary pancreatic cells. The second and third images in the figure are expected to represent NADH and FAD respectively, but the very bright spots in the images are consistent with neither. NADH and FAD must be found in every cell. The bright spots observed likely correspond to another fluorophore such as lipofuscin or ceroid.

The AF microscopy study demonstrates an instrument and technique for imaging cellular tryptophan AF. The images exhibit label-free visualization of pancreatic cells unique from what has been published previously. Modulation of protein with a proteasome inhibitor
confirmed that 280 nm excited AF intensity responds to changing protein levels.

Discrimination between normal and cancerous cells must still be established to use these AF images for diagnostic evaluation. Simple protein content alone may not be sufficient for diagnosis but additional information might be gained by refining the measurement either through alternate filter selection or by the acquisition of time resolved data. Combination of image data from multiple excitation wavelengths may also lead to an improved diagnostic for evaluating cells obtained by needle biopsy.

![Figure 3. Primary human pancreatic exocrine cells in short term culture. Images from left to right: 280 nm excited autofluorescence, 340 nm excited autofluorescence, and 440 nm excited autofluorescence.](image)
CHAPTER 3: FUTURE WORK

3.1 Future Outlook for Research Objective #1

The AF imaging study using 365 nm excitation and eight different emission bands showed diagnostic value in distinguishing normal and cancerous ovaries although specificity was limited to approximately 70%. Analysis revealed that only two principal components were needed to capture 99.6% of the variability in the data set. This indicates that similar performance could be achieved by collecting images in only two emission bands. Based on inspection of the second highest scoring principal component, it may be effective to choose one emission band above 450 nm and one below 450 nm. However, the effectiveness of an ovarian imaging instrument relying on a single excitation wavelength at 365 nm appears to be limited. Consider the jack knife analysis (100% sensitivity, 71.5% specificity) performed on the set of training data.

In order to increase specificity beyond 71.5%, while maintaining very high sensitivity, other information must be collected. A fluorescence spectroscopy study performed previously in our laboratory found the UV excitation wavelengths producing the most significant ability to distinguish normal, benign, and cancer ovarian pathologies were 270 nm and 340 nm. It appears that further study of these two wavelengths is warranted. One would first perform additional jack knife analysis on the identical training data used for the current study (complete excitation emission matrices are available) to determine how much diagnostic ability might be gained.

The diagnostic analysis up to this point has focused on distinguishing pathologically normal and cancer ovaries. Actual diagnostic use for ovarian cancer screening in high risk
women would also include assessment of benign pathologies such as endometriosis and various benign neoplasms. It would be desirable for the technique to distinguish a benign condition from a normal or cancer ovary, although it is not required. Initial study showed that benign neoplasms were designated as normal in a binary (normal or cancer) classification whereas endometriosis ovaries were designated as cancer in the same binary classification.\(^\text{17}\) It would be interesting to see how effective classification into four groups (normal, endometriosis, benign neoplasm, and cancer) would be.

Pixel by pixel diagnostic tissue mapping indicated significant heterogeneity in AF collected across the surface of an ovary which interfered with classification. This variation in signal will need to be characterized in order for imaging to prove more useful than point spectroscopic measurements. A necessary step will be to collect AF images from at least a small number of ovaries and follow with carefully documented sectioning or biopsy and spatially coordinated histopathology. Variation in AF signal can then be correlated to microstructure to determine how much is due to variation in absorbers (hemoglobin) versus variation in pathology (cellular/nuclear density, extracellular variation). Effects of variation in surface geometry may be studied by creation of phantoms.

For practical in vivo use, the AF imaging technique must be implemented in an endoscope and utilize a short exposure time (~0.1 s) to reduce motion artifacts. Investigation of the additional excitation wavelengths (270 & 340 nm) should be performed prior to construction of an endoscope as the results will help determine its design. The multispectral imager developed as part of the second specific aim of this dissertation is suitable for evaluation of these wavelengths.
Ultimately, sensitivity in detecting cancer would be the most important factor in determining acceptability of the technique for use in screening of high risk patients. A technique with high sensitivity but imperfect specificity would be preferable to blind removal (0% specificity) of the ovaries (the current preventative standard in older high risk patients). However, in order to prove very high sensitivity, a very large dataset is needed which includes not only late stage but also early stage ovarian cancers. Such a study will be prohibitive in time and cost requirements. A separate study in our lab (involving spectroscopic probe measurements) did yield high sensitivity of 92% and specificity of 87% in classifying malignant and non-malignant ovarian biopsies.\textsuperscript{18} It should be noted, however, that several excitation wavelengths and parallel factor analysis were utilized to obtain these results. Safe exposure levels of the ovaries to UV must also be investigated. Follicles of the ovary are most susceptible to UV.

Further study of AF ovarian cancer imaging will reveal how effective the diagnosis can be. The potential impact is limited by the fact that the technique requires minimally invasive surgery which can only be justified in high risk women; however, competing technologies such as endomicroscopy have the same limitation. It may be that improvement of the technique specificity by incorporating a second excitation wavelength would improve funding opportunities and drive the technique closer to adoption.

3.2 Future Outlook for Research Objective #2

In the AF imaging study of human colon specimens, six excitation wavelengths from 280 to 440 nm were investigated for contrast between lesions and normal colonic wall. The image with greatest and most consistent contrast was F440. Despite influence of FAD
fluorescence, the dominant contrast mechanism of F440 has been shown by others to be collagen AF from the submucosa [which is reduced due to either thickening of the mucosa at the lesion site\textsuperscript{38} or remodeling of the extracellular matrix (ECM).\textsuperscript{39}]

An attempt was made to improve contrast by utilizing additional fluorophores (e.g. F280 images to access tryptophan). However, F280 images did not confirm a previously observed doubling of tryptophan AF in cells harvested from colon cancer as compared with cells harvested from adjacent normal tissue.\textsuperscript{31} Typically a reduction in F280 lesion AF was observed and suspected to be due to greater hemoglobin absorption in the lesion. F280 images compensated for this effect (F280/R555), often reversed the observed AF reduction but remained low in contrast. Furthermore, efforts to combine tryptophan signal with F440 for contrast enhancement (such as ratios R1, R2, R3, R4, R5, R11) were of limited success, often producing high general contrast due to F440 but degraded image quality.

Attempts were made to utilize contrast from a third fluorophore, NADH, as captured by image F340. This image often appeared dominated by collagen AF as well as hemoglobin absorption, as evidenced by low intensity at lesions compared to the surrounding normal. Combining F340 and F440 in ratio images R27 and R15 produced improvement, especially in a minority of cases where F340 intensity was higher in the lesion than in the surrounding tissue. This resulted in a significant number of R27 and R15 images with better lesion contrast than F440 alone.

Of the six reflected light images between 370 and 555 nm, the image with greatest and most consistent contrast in LCLs was R555, the longest reflectance wavelength measured. This is not readily explained by hemoglobin absorption, since higher absorption occurs between 400 and 440 nm. The green light penetrates more deeply with decreased
scatter and less absorption. Therefore, unique scattering properties of the ECM at the basement membrane may produce this result. Ratio image R30 combined the low contrast image R480 with R555 to create several striking images. Such a ratio compensates for nonuniform illumination and geometrical effects.

This study of label-free spectral imaging in the colon confirmed the value of a blue excited autofluorescence image. No other spectral image tested rivaled F440. This indicates that simply combining other images studied with F440 may only result in incremental improvements.

R27 might be the most significant contribution of this study, as it shows how a UV wavelength can be used to improve lesion visibility in the colon. The ratio image $R_{27} = F_{340}/F_{440}$ works extremely well in a subset of low visibility lesions, yet in other lesions, F440 works better alone. The observation reminds me of one several years ago noting that some colonic lesions produce significant porphyrin fluorescence (red emission) when excited in the blue.\textsuperscript{38} The first AF colonoscope (Xillix’s LIFE-GI) exploited this. In both cases the impact is limited by the fact that only a subset of lesions displays these characteristics which enable detection. However, it remains to be seen how much has been gained with the discovery of R27. Collection of additional specimen images will confirm what type(s) of lesion are detected by this image.

Going forward with this research, I see a few areas for potential improvement. First, an effort should be made to uncouple the NADH and collagen signals in F340. It appears the reason some R27 images work so well is that NADH is high enough to dominate the collagen signal. If collagen and NADH fraction can be separated, F340 might provide useful metabolic related contrast in a greater number of lesions. Parallel factor analysis is a method
that has been used previously in the lab to uncouple signals but may require better spectral resolution than system images currently represent. An alternate method would be the use of 320 nm and 340 nm excitation images at two or more emission bands. Tissue phantoms including collagen and various concentrations of cells would need to be built and imaged.

It may be worthwhile to continue investigating contrast due to tryptophan, but the emphasis should shift to acquiring alternate types of images discerning different types of tryptophan. Complementary information can be extracted using one image consisting of approximately 300 to 340 nm emission and one with 340 to 400 nm emission. Tryptophan buried in the hydrophobic core of protein is known to have blue-shifted emission, while the opposite shift is true for tryptophan exposed to polar environment. (In order to image emission below 340, the shortpass filter mounted at the camera lens will need to be removed or replaced and the 280 nm excitation filters adjusted to ensure minimal NIR leakage.)

Alternately, polarization control has been used to distinguish fluorescence from fluorophores in different molecular binding configurations (e.g. NADH40). Fluorescence anisotropy imaging of tissue has not been thoroughly investigated. A review of techniques was given by Jameson.41

Finally, I have discovered that it is not trivial to combine multiple (2 to 3) images in such a way that the useful contrast of an object in one image is not degraded by combination with another image. For example, if one divides an image of positive contrast by a matching second image of negative contrast, the resulting image has contrast greater than either original image. However, the first and second image may both possess positive contrast. Division of the two images then produces an image with lower contrast than either original image. Therefore, when the observed contrast is variable (and unpredictable), a traditional
ratio image (A/B) is sometimes beneficial and sometimes detrimental. The same limitation applies when combining two ratio images through division, e.g. (A/B) / (C/D), or when placing two or three ratio images in different color channels of a pseudocolor RGB image.

In future work (using multiple images related to different fluorophores), it would be useful to develop a logical method of combining the images which is “lossless” in terms of contrast. Such a method would be equally applicable to creating gray scale ratio images and false color RGB images. It would also be ideal for combining two or three independent ratio images in a single, best-contrast image. Personal efforts indicate this “lossless” method of combining images is a significant undertaking with no easily implemented solution. Unless such a method can be developed, a dual image display is needed for simultaneous visual monitoring of independent ratio images like R27 and R30. Although dual grayscale images provide better theoretical color contrast, a dual RGB image display will likely be more marketable.

3.3 Future Outlook for Research Objective #3

The AF microscopy study focused on sub-300 nm excitation to produce several key findings about tryptophan imaging which may be built upon in future work. From a device standpoint, it was demonstrated that a transillumination microscope configuration can be useful, especially in the case that a specialized objective with good sub-300 nm transmission is not available. A quartz microscope slide is needed in this configuration, but we successfully used a standard glass coverslip to suppress excitation light.

The drawback of the configuration used in the present study was the need to invert the cell coverslip. With the coverslip inverted, creation of a fluidic chamber was needed to
avoid compression of the cells between coverslip and microscope slide. This led to problems with air bubble formation and difficulty in replacing media for organelle stain experiments. The next generation of this system should utilize an open air (rather than closed chamber) configuration and a larger volume of cell sustaining media. This would be achieved in one of two ways. The current upright microscope and transillumination setup could be used, but with no coverslip and the objective immersed directly in the cell media. Cultured cells for study could be grown directly on quartz slides or in cell culture dishes with quartz base (http://www.agarscientific.com/culture-dishes.html). Alternately, an inverted microscope could be used and light brought in from above the sample. This arrangement would keep the objective from getting dirty. Cells could be grown on standard coverslips.

It is worth noting that the ideal instrument would allow collection of tryptophan emission below 340 nm. Since the tryptophan emission peak can be as low as 330 nm (polarity of local environment results in a shift), it would be desirable to obtain a microscope objective with high transmission down to 310 nm.

One of the key cellular imaging results was the group of experiments demonstrating that sub-300 nm excited AF increases with cellular protein levels. A 40% increase in AF signal was observed in cells treated with Velcade for 32 hours. To my knowledge, no such result has been previously demonstrated. The next step is to characterize differences between normal and cancer cells. It could be very difficult to distinguish cells based on absolute AF intensity, as careful system calibration is required and a 40% increase is relatively small. One might also suppose that a certain amount of protein level variation in a cell population is normal. Diagnosis based on a ratio measurement might produce more consistent results and reduce calibration requirements. The most useful ratio must be determined through study but
could include NADH emission versus tryptophan emission or be based on two emission bands both excited at 280 nm. Alternately, polarized fluorescence images might reveal more significant differences between normal and cancer cells. The goal in using multiple emission bands or multiple polarization configurations would be to target and separately image subgroups of tryptophan such as residues buried within large proteins or residues exposed to a more polar environment. Either of these may provide greatly improved diagnostic information.

In cultured cells it was found that the nucleolus often produced bright sub-300 nm excited AF. That finding could be significant due to the fact the imaged cancer cell lines displayed this characteristic, but the immortalized normal cell line did not. AF of the nucleolus should be studied in additional cell lines to see if the trend continues. The nucleolar AF may be a measure of ribosome biosynthesis, a process which becomes dysregulated in some cancers.

Currently, the goal for this AF technique is quick diagnosis of biopsy cells obtained through fine needle aspiration (FNA). Therefore, an FNA-compatible protocol must be developed. In FNA, small amounts of cells and fluid are collected with a needle and syringe and deposited either in a vial of fixative or directly on a slide where a smear is created. Preliminary studies of the tryptophan AF have used living cells obtained both through cell culture of established lines and isolation of primary pancreatic cells through tissue dissociation. Assuming that viability of FNA-obtained cells is needed (although this is currently unknown), the contents of the needle and syringe should first be expelled in a tube of warmed Hank’s balanced salt solution (HBSS) (or other sustaining media) and needle rinsed in same tube. Next, cells might be spun down and deposited directly on a quartz slide
using the popular Cytospin technique. A fresh drop of HBSS on the slide and coverslip on
top would maintain the cells during AF diagnostic imaging.

This description of an FNA-compatible protocol is hypothetical and incomplete
without testing. The decision to deposit the FNA product in a tube and then perform
Cytospin (instead of utilizing a simple smear technique) is made because FNA is expected to
result in a relatively small number of cells. A wash step may also be needed prior to
Cytospin. Maintaining cell viability could prove difficult, and if highly viable cells are
needed, it may be necessary to incubate the biopsied cells in a complete culture media for a
few hours (or more) before imaging. This would reduce the speed of the technique and, thus,
its value as a quick diagnostic. If noncellular debris is abundant in the FNA samples, then
one or more steps will need to be added to the protocol for its removal. An AF technique
compatible with fixed cells would be highly desirable because any delays in executing the
protocol will impair cell viability.

In order to develop an effective diagnostic test, it appears that further exploration of
the new AF technique is needed using cultured cells. I would put emphasis on investigating
other types of tryptophan AF images (multiple emission band, polarized AF, or time resolved
AF) and ratios as discussed. Nucleolar AF may be useful diagnostically but is perhaps not
very practical, as a bright nucleolus could be difficult to see in rounded up cells compared to
its visibility in flattened cultured cells. Ratio images can be constructed from existing data,
but I expect them to be of low quality and limited utility due to the weak NADH AF. My
first priority would be getting multiple tryptophan emission band images and polarized AF
images, as these are minor variations on the existing system with similar potential to improve
diagnosis of cells. Lifetime imaging requires a significantly more expensive system which
has less potential impact because of the cost. The largest impact on FNA cell analysis would be created by a technique that can be made effective on fixed cells, therefore reducing the care and skill needed to perform the AF protocol. If diagnosis with tryptophan AF proves successful, then finding a compatible fixative should become a priority.
REFERENCES


APPENDIX A - WIDE-FIELD SPECTRAL IMAGING OF HUMAN OVARY AUTOFLUORESCENCE AND ONCOLOGIC DIAGNOSIS VIA PREVIOUSLY COLLECTED PROBE DATA
Wide-field spectral imaging of human ovary autofluorescence and oncologic diagnosis via previously collected probe data

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Wide-field spectral imaging of human ovary autofluorescence and oncologic diagnosis via previously collected probe data

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Abstract. With no sufficient screening test for ovarian cancer, a method to evaluate the ovarian disease state quickly and nondestructively is needed. The authors have applied a wide-field spectral imager to freshly resected ovaries of 30 human patients in a study believed to be the first of its magnitude. Endogenous fluorescence was excited with 365-nm light and imaged in eight emission bands collectively covering the 400- to 640-nm range. Linear discriminant analysis was used to classify all image pixels and generate diagnostic maps of the ovaries. Training the classifier with previously collected single-point autofluorescence measurements of a spectroscopic probe enabled this novel classification. The process by which probe-collected spectra were transformed for comparison with imager spectra is described. Sensitivity of 100% and specificity of 51% were obtained in classifying normal and cancerous ovaries using autofluorescence data alone. Specificity increased to 69% when autofluorescence data were divided by green reflectance data to correct for spatial variation in tissue absorption properties. Benign neoplasm ovaries were also found to classify as nonmalignant using the same algorithm. Although applied ex vivo, the method described here appears useful for quick assessment of cancer presence in the human ovary. © 2012 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.JBO.17.3.036003]

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1 Introduction

Ovarian cancer is a particularly deadly disease that, as of 2010, had a five-year survival rate of 46% in the United States. 1 Currently, no screening test is capable of consistently detecting the disease at the early, localized stage. 2 For this reason, only 15% of all ovarian cancer cases are diagnosed at the localized stage in which the disease is highly curable. 2 It is well documented that approximately 90% of ovarian cancers arise in the epithelium, 3 or spatial layer of the ovary, which validates the feasibility of laparoscopic surgery for early ovarian cancer screening.

One promising approach for disease detection and localization in numerous organ sites has been spectroscopic evaluation of endogenous tissue contrast using both fluorescence and reflectance-based techniques. 4 Autofluorescence spectroscopy has been applied to distinguish malignancy in tissues of the human lung, breast, skin, oral cavity, cervix, gastrointestinal tract, brain, bladder, and ovary. 4 These studies of native tissue fluorescence, including both steady-state and time-resolved measurements, have been extensively reviewed. 5-8 Diffuse reflectance spectroscopy (DRS) has likewise been applied to all the tissues mentioned above, with emphasis on the skin, 9-10 breast, 11-15 brain, 16-18 and cervix. 19-21

While “point” spectroscopy techniques based on autofluorescence, diffuse reflectance, or some combination of the two have shown promise for noninvasive optical biopsy, these methods have not gained common clinical usage, let alone supplanted the standard needle biopsy or excisional biopsy. The spectroscopic diagnostic devices that have reached commercialization 22-24 have combined both spectral and spatial information through spectral imaging—also called "multispectral" or "hyperspectral" imaging. This method is realized either through the collection of images at various wavelength bands or by the acquisition of spectra at a multitude of point locations. Recently, Balas 25 reviewed the status of spectral bioimaging. Spectral imaging of autofluorescence seems to have found its niche as a means of improving the visualization of neoplastic lesions on the cervix, 23 in the mouth, 26 and, endoscopically, in the lungs 26 and the gastrointestinal tract. 24

Another promising optical biopsy method for cancer is the time-resolved version of autofluorescence imaging, known as fluorescence lifetime imaging (FLIM). In this method images are constructed from spatially resolved fluorescence lifetime measurements. Fluorescence lifetimes, like autofluorescence intensity and spectral shape, have been proven useful in determining tissue pathological state. FLIM endoscopes and microscopes have been developed for distinguishing cancer in the lungs, 27 skin, 28 oral cavity, 29 and brain. 30 FLIM provides benefits over steady-state fluorescence imaging that include insensitivity to illumination intensity and reduced sensitivity to tissue optical properties. While FLIM is a promising imaging modality, it also has drawbacks including increased instrumentation complexity and measurement acquisition time. FLIM will not be discussed in detail here as the spectral imager of this study incorporated only steady-state fluorescence acquisition.

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Speaking again of spectral imaging in general, clinicians typically use spectral images together with their own experience to select tissue locations to biopsy and analyze with standard histology, the gold standard for diagnosis. However, automated analysis of tissue spectral images renders optical diagnosis more objective and reduces the need for an experienced clinician with optical knowledge to perform the evaluation. For example, tissue images can be transparently overlaid with false-color maps indicating a simplified optical diagnosis. Several groups have demonstrated spectral image analysis enhancements with varying degrees of automation.  

In the study described here, we have applied a 365 nm-excitation multispectral imager (MSI) to freshly resected whole human ovaries, collecting autofluorescence images in eight different spectral bands spanning the 400 to 640 nm visible range as well as reflectance images in four different visible spectral bands. To our knowledge, this is the first wide-field spectral imaging study of human ovary autofluorescence of this magnitude. We diagnostically map large areas of tissue by utilizing linear discriminant analysis and a library of previously-collected fluorescence probe spectra (from ovarian biopsies of histology-confirmed diagnosis). The incorporation of training data from a second instrument was necessary due to the impracticability of extensive biopsy of all the ovaries imaged in the study. We elaborate a process for transforming the probe spectra, enabling one-to-one comparison with MSI spectra. Sensitivity of 100% and specificity of 51% were obtained using fluorescence data alone for the diagnostics. Specificity was increased to 69% after correcting autofluorescence data for variation in tissue optical properties. The correction used was a division of measured autofluorescence by green reflectance data also collected by the MSI.

2 Materials and Methods

2.1 Clinical Studies and Ovarian Pathology

Multispectral fluorescence images of 49 whole human ovaries from 30 patients were collected ex vivo through a clinical study at the University Medical Center of the Arizona Health Sciences Center at the University of Arizona in Tucson. The study (A02.31/OB/GYN) was approved by the Institutional Review Board (IRB) of the University of Arizona, and informed consent was obtained from each patient before tissue imaging occurred. All study participants were women undergoing planned oophorectomy either for disease treatment or as prophylaxis. The average patient age was 58, and 25 of the 30 patients were postmenopausal.

In this study an MSI with 365-nm illumination was used to perform fluorescence spectroscopy. The goal was to define regions of abnormal tissue as well as any tumor margins that might be present in images by observing spatial variations in autofluorescence. A central assumption was that tissue pathology could vary across a single wide-field image. It was, therefore, desirable to have histopathological results from many small tissue areas in order to validate diagnostic mapping; however, time restrictions of the pathologist prevented each ovary from being completely sectioned and microscopically analyzed. Instead, the standard gross pathology assessment was performed; that is, one to three sections of each ovary were made and analyzed to determine a diagnosis.

In the absence of complete sectioning and histopathology of the whole ovaries in the imaging study, we elected to utilize a second dataset collected previously as part of a nonimaging (single-point) ovarian autofluorescence study (#02-113/OB/GYN). In that study, small excisional biopsies were collected from each ovary at the site of each probe-based spectral measurement and sectioned for microscopic analysis. This single-point spectral dataset and corresponding histopathology were used to train a classifier that we applied to the tissue spectral images collected by the MSI.

For our purposes, it was useful to group all of the numerous ovarian pathologies into four general categories: normal (no structures except stroma, epithelium, corpus albicans, and corpus luteum), benign neoplasm (abnormal growth without invasive areas), cancer (invasion of carcinoma into the ovary), and endometriosis (growth of both endometrial glands and stroma on the ovary). Included in our analysis are 92 sets of multispectral images collected from 24 ovaries of normal pathology, 10 sets of multispectral images collected from three ovaries of benign neoplasm pathology, 12 sets of multispectral images collected from two ovaries of cancer pathology, and 30 sets of multispectral images collected from six ovaries of endometriosis pathology. Excluded measurements are detailed in Sec. 3.7. The diagnostic classifier was trained using only ovaries of normal and cancer pathological diagnoses but then applied to ovaries of all four aforementioned pathologies. Histopathology was used as the diagnostic gold standard.

2.2 MSI

A multispectral imaging system (SEAtreat, Apogen Technologies, Inc., now QinetiQ North America, San Diego, CA), henceforth referred to as the MSI, was used for automated acquisition of both fluorescence and reflectance images of whole ovaries. The imager, whose simplified optical layout is presented in Fig. 1, has a fixed focus at a working distance of 30 cm, field-of-view 40 mm × 33 mm, and resolution 580 × 475 pixels. The device acquires four spatially registered images simultaneously on a single charge-coupled device (CCD) using a patented technique, Quad-Prism Aperture.
Splitting (QPAS). Fluorescence illumination is provided by the MSI using a short-arc mercury lamp (LC6 series, Hamamatsu, Bridgewater, NJ) filtered to include only the ultraviolet line emission at 365 nm and coupled to a fiber-optic bundle that carries the light from the lamp to the imaging head. Ultraviolet (UV) light is projected onto the tissue at a power density of 18 mW/cm² and excites a broad spectrum of autofluorescence, which is collected, divided into four parallel optical paths (each uniquely band-pass-filtered), and imaged onto four quadrants of a single CCD. A motorized filter wheel allows rapid exchange of the bandpass quad-filter, and thus a total of eight images are collected by two consecutive exposures of the CCD. The eight fluorescence emission bandpass filters effectively cover the spectrum from 400 to 640 nm with little overlap. The filter center wavelengths are 420, 440, 455, 485, 510, 530, 560, and 600 nm.

For reflectance imaging, a halogen bulb provides linearly polarized white light illumination at a power density of 8 mW/cm². A different set of quad-filters is rotated into the optical path for collection of each set of reflectance images. Each reflectance quad-filter has blue, green, red, and near-infrared quadrants and is masked by a polarizer whose transmission axis is either crossed or parallel in comparison to the polarization of the illumination. Light that is reflected specularly at the tissue surface is highly attenuated by the crossed polarizer.

2.3 Multispectral Imaging Procedure

Oophorectomy was typically laparoscopic, but in some cases an open surgery was performed. Whole ovaries and attached fallopian tubes were severed via electrosurgery. Removal of tissue from the body occurred sometimes through one of the laparoscopic incisions (after placement of ovary in a protective plastic bag) and sometimes vaginally (in coordination with a hysterectomy). Each ovary was bathed in room temperature saline solution and transported in a metal bowl. Care was taken to always grasp the ovaries by the fallopian tube to avoid damage to the fragile ovarian epithelium. Ovaries were taken to a darkened room and placed on a plastic Petri dish for imaging. A solution of phosphate-buffered saline (PBS), glucose, and L-glutamine was used to rinse blood from the ovarian surface and to maintain moisture during imaging. Imaging typically began within 30 min after loss of blood supply to organ and required 5 to 8 min from start to finish. In all cases, imaging began less than 60 min after loss of blood supply to organ. Data collection within 90 min of biopsy has been shown to minimize difference of ex vivo fluorescence measurements compared to in vivo. Typical integration times were 100 ms for fluorescence images, 5 ms for parallel-polarization reflectance images, and 13 ms for crossed-polarization reflectance images.

2.4 Single-Point Spectral Data

Single-point autofluorescence data were collected from fresh 5- to 10-mm-sized ovarian tissue biopsies as part of a previous study also at the University Medical Center at the Arizona Health Sciences Center (#02-113/OB/GYN). In total, 249 single-point spectra were collected from 49 patients, with 186 of the spectra being collected from ovarian tissue deemed histopathologically normal, 25 being collected from tissue deemed cancer, 10 collected from tissue deemed endometriosis, and eight from tissue deemed benign neoplasm. The remaining 20 measurements could not be used because pathology was unavailable.

The device used to collect single-point spectral data was previously described, briefly, a spectrofluorometer (FluoRoLog 3-22, JY Horiba, Edison, NJ) using double excitation and emission spectrographs coupled to a custom fiber-optic probe that was used to interrogate a tissue spot of 800-micron diameter. The six-around-one fiber configuration probe was positioned in contact with the surface epithelium allowing the weight of the probe to contact the tissue. Spectra were recorded from three to four locations on each biopsy. Biopsies were placed in chilled Roswell Park Memorial Institute (RPMI) culture medium (Media Tech, Herndon, VA) and transported to the optics laboratory. Typically, fluorescence measurements were performed within 60 min after removal. Subsequently, tissue was fixed and processed with standard histological procedures. A pathologist examined each ovarian biopsy at University Medical Center, and pathology results were obtained.

The collected single-point data was in the form of an excitation emission matrix (EEM) with 14 different excitation wavelengths from 270 to 550 nm. Data were calibrated for variations in excitation power as well as spectral sensitivity of the detector.

2.5 Single-Point Spectral Data Conversion Process

The single-point autofluorescence data EEM are of relatively high spectral resolution, sampled every 5 nm, while the MSI data are low-resolution “spectra,” which include only eight measurements over the same emission range (see Fig. 2). The eight-point MSI spectra are not spectra in the strictest sense because each spectral image is weighted by the system’s spectral sensitivity, which varies with the transmission characteristics of the optical filters used for collection. For one-to-one comparison of
these two datasets, we needed either to weight the single-point EEM data with the spectral sensitivity characteristics of the MSI yielding eight-point filter-weighted spectra or to convert the eight-point MSI spectra into true spectra.

Mathematically, it was easier to transform the single-point EEM spectra to the eight-point spectral space of the MSI. We accomplished this by multiplying the single-point spectra by the measured sensitivity characteristics of the MSI at each filter configuration. This filter-weighting process is illustrated in Fig. 3. Notice that each horizontal line of data in the single-point EEM corresponds to a different excitation wavelength. We utilized only the portion of the EEM corresponding to 365-nm fluorescence excitation (denoted with black line and arrows in Fig. 3), as this was the only excitation wavelength of the MSI.

2.6 MSI Data Pre-Processing and Calibration

Image processing and analysis was performed using MATLAB (The MathWorks, Natick, Massachusetts). Each set of raw image data from the CCD was 1024 × 1280 pixels and contained four images—one 512 × 640 pixel image from each quadrant. A corresponding set of dark images was captured for each image set. Manufacturer-provided code was used to perform image pre-processing as follows. The first step involved breaking the composite image into four separate images. Then spatial registration of the images and correction of distortion were performed. Next, dark subtraction and flat field illumination correction were applied. Scale factors were used to account for differences in integration time.

We implemented additional pre-processing steps to the original MATLAB code. Background subtraction of a nonfluorescing standard was applied to correct for device autofluorescence. The subtracted background measurement was taken in a dark room with imager viewing a distant, nonfluorescent black cloth. The power level of the UV source was measured daily with a power meter, and any variation was corrected for in the images via a scale factor.

The MSI data and single-point spectra compared in this study were collected by two different devices utilizing different means of wavelength selection and different detectors. As a result, calibration for the spectral response of each system was required. To eliminate device-dependent differences in collected spectra, solid and liquid fluorescence standards were imaged with the MSI and also measured with the spectrofluorometer. The solid standards used were tetraphenylbutadiene (TPB) and rhodamine B (Starna Ltd., Hainault, UK), while the liquid standard was fluorescein in a quartz cuvette (Molecular Probes, Eugene, OR).

For a one-to-one comparison of the measured eight-point fluorescence spectra to the known spectra provided by the manufacturer of the fluorescence standard, it was necessary to weight the known fluorescence spectrum of each standard with the spectral sensitivity characteristics of the MSI (through the process illustrated previously in Fig. 3).

Division of the calculated eight-point fluorescence emission spectrum of a fluorescence standard by its measured eight-point spectrum yielded a set of correction factors for the absolute spectral response of the MSI. Applying these multiplicative correction factors to the spectral images captured by the device allowed calibrated fluorescence emission spectra to be extracted from the collection of uniquely filtered, spatially registered images.

2.7 Intensity Matching of MSI Data to Single-Point Data

Finally, to compare the single-point emission spectra and the MSI spectra, only intensity equalization was lacking. We aimed to calculate a single scale factor that would adjust the fluorescence intensities collected by the spectrofluorometer to be on the same level as those collected by the MSI. Instead of comparing measurement intensities of fluorescence standards between the two systems, we found that calibrating the intensity distributions of all measurements made on ovaries deemed normal by histopathological evaluation was an appropriate procedure.

The single-point measurement intensity histogram (composed of 186 measurements on normal tissue) and MSI data intensity histogram (composed of 92 measurements on normal
tissue), both shown in Fig. 4, were produced as follows. Single-point measurements were first converted to eight-point spectra as described previously. Only 455-nm measurement intensities were included in the histogram because they typically represented the maximum of the spectrum. For the MSI data, a unique region of interest (ROI) was first manually selected in all images. (In order to lessen the effect of camera view angle on image intensity, the ROIs were selected to include only tissue approximately normal to the camera). Then, for each image, the 455-nm-filtered intensity was averaged over the ROI pixels. These average pixel intensities were used in constructing the intensity histogram of MSI data.

Two methods of intensity equalizing the datasets were tested. In the first method, a ratio of the means of the two intensity distributions was used as a scale factor. For the second method, a gamma distribution was fitted to each intensity histogram, and the scale factor used was a ratio of the peak intensity of each fitted gamma distribution.

2.8 Classification Method and Optimum Preparation of Data

Linear discriminant analysis (LDA) is a statistical method useful for predicting whether a measurement belongs to one class or another, and to perform LDA, a set of training measurements of known class is required. In our case the chosen measurement classes were normal and cancer, and our library of single-point spectra and associated gold-standard pathologies served as the training data. In LDA, each measurement can be considered to lie somewhere in an N-dimensional “measurement space.” Training measurements define the distribution of each class within the N-dimensional space. Given a measurement, LDA calculates which class (and distribution) the measurement most likely belongs to. In this study, LDA was performed by using the “classify” function included in the MATLAB statistics toolbox.

Before classification via LDA, it was desirable to reduce data dimensionality. N. Smaller N reduces computational load and, up to a point, increases the effectiveness of LDA. We applied principal component analysis (PCA) to the data sets by calculating the covariance matrix of the data and decomposing it into eigenvectors and eigenvalues. The highest scoring eigenvectors, or principal components (PCs), represent a basis for a lower dimensional space that measurements can be mapped to. PCA maximizes the variance of the data that can be represented by a given number of PCs.

The appropriate number of PCs to use for LDA was determined by performing jackknife analysis on the set of training measurements and varying the number of PCs used for the measurement space. One measurement at a time was removed from the dataset and PCA applied to the remaining measurements. LDA was then used to classify the removed measurement, and the process was repeated for each measurement, always replacing one measurement and removing another. Sensitivity and specificity of the classification were calculated and used to select an appropriate number of PCs and thus the final data dimensionality. Jackknife analysis was similarly used to determine whether classification would be more effective using unnormalized or normalized data.

2.9 Diagnostic Tissue Mapping

Many choices exist for effectively displaying multispectral image information. Each single specific wavelength image can be observed in gray scale, or three specific wavelength images may be observed together in a false-color red, green, and blue (RGB) image. However, eight specific wavelength images cannot be displayed in full spectral detail in a single image of three-color channels.

The approach we took was to statistically analyze the spectral data at each image pixel and then visually display the results in a binary image. LDA was applied to classify the measured spectrum from each pixel in an image ROI. The LDA training set consisted of a library of 211 single-point spectra. Prior to classification, PCs calculated from the training set were used to map the test spectra to a new measurement space. For every multispectral image set, a diagnostic tissue map was produced by overlaying the ROI of a specific wavelength fluorescence image with color-coded pixels. Pixels that classified as normal were colored green, and pixels that classified as cancer were colored red, as will be seen in later figures.

3 Results

3.1 Multispectral Imager Calibration

Correction factors for the absolute spectral response of the MSI were calculated as described in Sec. 2.6. Results from measurements on three relevant fluorescence standards are compared in Fig. 5. The plotted data illustrates that longer wavelength bands must be scaled up in intensity relative to images at shorter bands. The system sensitivity therefore appears to be lowest at the longest wavelengths imaged. Comparison of the three normalized curves in Fig. 5 shows excellent agreement between the fluorescence measurements of TPB and fluorescein except at
the longest wavelength emission filter. However, both TPB and fluorescein fluoresce weakly at 600 nm, while rhodamine B fluoresces significantly there. For this reason, the first seven correction factors are selected from TPB results, but the final correction factor is chosen to match the rhodamine B results.

3.2 Inter-Device Spectral Shape Correction

Using gross histopathology to group fluorescence spectra, we compare the average MSI-collected spectra of normal, cancer, and benign tissues, where the benign group includes both benign neoplasm and endometriosis. Each average spectrum is calculated by taking the mean of each spectrum from all ROI pixels of each image and then averaging over all the tissue images of the same histopathology. Results are shown in Fig. 6(a) with the average spectra from the single-point device shown in Fig. 6(b) for reference. In both the imaging study and the single-point study, the average fluorescence intensity from normal ovarian tissue is much higher than from cancer tissue. In the imaging study, the average benign fluorescence was highest of the three measurement groups. Although inconsistent with the single-point study result, this finding agrees with that of Brewer et al., and we note that the benign group of measurements was small in the single-point study.

The average normal spectrum acquired with the MSI [Fig. 6(a)] displays a different shape compared to the single-point device spectrum [Fig. 6(b)]. The fluorescence peak is red shifted, and the relative intensities at the longest wavelengths are enhanced. Although single-point measurements were collected from a separate set of normal ovaries, we expect the average fluorescence properties of the tissues to be very similar. Gebhart et al. published a comparative study of single-point and spectral imaging fluorescence measurements from tissue phantoms and observed redshifted spectra; the effect they observed was, however, less pronounced. Dividing the average spectra of the two devices yields monotonic functions, shown in Fig. 6(c), which are similar for spectra of both normal and cancer tissues. The dissimilar device spectra could therefore stem from differences in the illumination-collection geometries of the two systems. To equalize the two datasets prior to classification, all the MSI spectra were shape-adjusted by multiplication with the normal curve shown in Fig. 6(c). Henceforth, we refer to them as shape-corrected spectra.

3.3 Correction for Spatial Variation of Tissue Optical Properties

We observed that the presence of blood or increased vascularity on or near the ovarian surface seemed to reduce measured autofluorescence and to interfere with classification. Tissue optical properties (absorption and scattering) affect both the
propagation of excitation light into the tissue and the ability of emitted autofluorescence to escape the tissue for collection by the imaging system. To correct for the absorption effects of blood and hypervascularity, we divided the fluorescence images by a green-channel cross-polarization reflectance image. Correction using blue and red reflectance images was attempted as well but produced less favorable results. The simple correction method we use has similarity to the one employed by Zeng, who divided skin fluorescence spectra by reflectance measurements at corresponding wavelengths as a first-order correction for tissue absorption and scattering effects. Our correction is also similar to the one used by Qu, who divided tissue fluorescence images by crossed polarization reflectance images captured at the excitation wavelength to correct for tissue absorption and geometrical effects. To avoid disturbing the average intensity level of the fluorescence images, we normalized each reflectance image to have an average value of one over the ROI prior to the division.

3.4 Optimum Preparation of Data for Classification

Jackknife analysis was performed on the training set spectra (single-point probe spectra) to determine whether classification of normalized spectra or unnormalized spectra would be more effective. The sensitivity realized using unnormalized data (100%) was much higher than when using data normalized by area (56%). Specificity realized using unnormalized data (71.5%) was comparable to that using data normalized by area (73.1%). Figure 7 illustrates the advantage of using unnormalized data. The unnormalized data display an obvious trend of lower-intensity cancer autofluorescence measurements and higher-intensity normal autofluorescence measurements. Normalizing the training data spectra by area emphasizes spectral shape; however, critical intensity information from measurement to measurement is lost in the process.

Figure 8 shows the two highest-scoring eigenvectors from PCA of the single-point training spectra. The first eigenvector, or principal component (PC), resembles the average eight-point spectrum of the training set. The second eigenvector allows lower (violet) or higher (blue, green, red) wavelength bands to be weighted more heavily. These two eigenvectors combined to represent 99.6% of the variance of the training data, which suggests that two PCs sufficiently represent the data.

To confirm the appropriate number of PCs for representation of the measurements, additional jackknife analysis was performed on the training set, and the results are displayed in Table 1. The combined sensitivity and specificity of classification was maximized when three PCs were used; however, two PCs were chosen for measurement representation because the lower number of PCs increases the model robustness.

3.5 Tissue Classification (Mapping) Via LDA

Autofluorescence images from the MSI were preprocessed, calibrated, and intensity equalized to the training set measurements as described previously. The eight-point spectra were then shape-corrected to match the training set spectra. Component loadings of both the test set and training set spectra were calculated based on PCA of the training set spectra (Fig. 8). Figure 9 shows the two classes of training set measurements plotted in the space of training set PC 1 and PC 2. The diagonal line designates the boundary for classifying a test measurement into the normal or cancer class. Sequentially, LDA was used to classify each ROI pixel of each tissue image into one of these two training set classes.

Figure 10 displays classification results from a single normal ovary with a bumpy surface. The RGB reflectance image

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**Fig. 7** (a) Filter-weighted unnormalized training data measurements show a trend of lower autofluorescence intensity in histopathology-confirmed cancerous biopsies. (b) Filter-weighted training data measurements normalized by area under curve show no obvious spectral feature distinguishing normal and cancerous biopsies.

**Fig. 8** Results of principal component analysis (PCA). The first eigenvector, or principal component (PC), represents the vast majority of the variance in the training dataset, and its shape closely resembles the average spectrum. These facts indicate that the dataset can be classified fairly well by considering only the intensity of a measurement. The second PC accounts for a small fraction of the dataset variance and can be used to emphasize portions of the spectrum lower or higher than 455 nm.
Table 1 Results of jackknife analysis on the training data using different numbers of PCs.

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PCs = principal components.

[Fig. 11(a)] shows abundant vasculature near the top and left of the image ROI, especially in the creases of the uneven tissue. Plots on the right side of the figure show distributions of the MSI data PC loadings (including only pixel data from inside the ROI) laid over distributions of the normal training set data PC loadings. Figure 11(b) shows PC loadings representing MSI spectra that are uncorrected for spectral shape as discussed in Sec. 3.2. These data do not overlap well with the training set. This is consistent with the previous observation that the training and test sets produced differently shaped average spectra. The color-coded classification map [Fig. 11(c)] shows that after spectral shape correction, 76% of pixels classify as normal tissue (pixels shown in green) and 24% like cancer tissue (pixels shown in red). Shape correction greatly improved overlap of the image data with the training data [Fig. 11(d)], and we observed a similar effect for spectra from other samples. When applying the correction for spatial variation in tissue optical properties, improved classification results were achieved [Fig. 11(e)]. Figure 11(f) shows that division of the shape-corrected fluorescence data by green reflectance data has shifted many of the ROI pixels from the cancer classification region at left of the decision boundary to the normal region at right. The total area covered by the cloud of data points is also reduced.

Presented in Fig. 11 are the classification results from imaging of a single cancer ovary. As seen in the reflectance image [Fig. 11(a)], this ovary had grown to an unusual size exceeding the camera field of view. Distributions of the image data PC loadings [Fig. 11(b), 11(d), and 11(f)] show that this ovary could be easily classified as cancer as image data lies clearly on the cancer side of the linear decision boundary. Shape correction and application of reflectance data both reduced classification uncertainty by moving the data distribution further left of the boundary. Maps of the classification results in Fig. 11(c) and 11(e) confirm complete success in identifying the ovary as cancer. It is seen also that, for this particular ovary image, correction with reflectance data was unnecessary.

An example of an image of a normal ovary that was difficult to classify with LDA is given in Fig. 12. This ovary had a significant amount of adipose tissue on its surface. The classification based only on shape-corrected autofluorescence [Fig. 12(b)] gives mixed results. One can see that regions with more blood tend to misclassify. Figure 12(c) shows that dividing by the green reflectance image generally improves the classification in areas with more blood but leads to misclassification in adipose areas. This shows a limitation of our simple method of incorporating green reflectance data. Dealing with adipose tissue seems to require an algorithm that considers red and/or blue reflectance as well. Presence of adipose tissue on the ovaries appears uncommon, however, as it was only encountered for one set of ovaries in this study.

3.6 Performance of Classification Mapping

As a measure of the effectiveness of the MSI and classification scheme in performing ex vivo ovary diagnosis, we designed criteria to allow calculation of sensitivity and specificity of our diagnostic algorithm on the multispectral data. If 90% or more of the ROI pixels in an ovary image classified normal, we called the result a normal classification of the ovary. Vice versa, if 90% or more of the ROI pixels in an ovary image classified as cancer, we called this a cancer classification of the ovary. For any other percentage classification of the pixels in an ROI, the classification was called “indeterminate.”

One hundred four images of 26 ovaries were classified for the first part of this study. From histopathology, 92 of these images (of 24 ovaries) were known from gross pathology to be normal, while 12 images (of two ovaries) were known to be cancer. The results of classification via LDA are presented in Table 2. Indeterminate classifications were treated the same as cancer classifications when calculating specificity and sensitivity. In all cases, intensity equalization was performed by scaling training set intensities using the ratio of mean 455-nm fluorescence of all normal training set measurements to normal test set measurements. The alternate method of intensity equalization based on the ratio of peak locations of fitted gamma distributions produced 30% lower specificity results and was abandoned. Jackknife analysis on the training data, Table 2, column one, represents an expectation of the highest sensitivity and specificity that can be achieved, as jackknife analysis on training data typically outperforms classification of a test set.

As shown in Table 2, sensitivity of 100% was achieved in all cases. We note, however, that two of 12 cancer images classified as indeterminate when data was not reflectance normalized, and this number improved to just one of 12 images when fluorescence measurements were divided by green reflectance.
Although the number of cancerous ovaries in the study was small, the high sensitivity of the method is supported by the 100% sensitivity realized in the jackknife analysis of the training data, which included a larger number of cancerous measurements. Specificity when using the shape-corrected fluorescence data was considerably lower than predicted by jackknife analysis (51.1% versus 71.5%); however, dividing the fluorescence data by green reflectance improved the specificity to 68.5%, which is comparable to the jackknife analysis. To judge the benefit of imaging at multiple emission wavelength bands, we also performed classifications using single fluorescence bands. One such band chosen to classify with was the single filter wavelength 455 nm, which represented the peak of the measured eight-point fluorescence spectrum. We also tested classification with a wide fluorescence emission band formed by summing all eight points of the measured spectrum. Both of these simple cases produced reduced specificity, justifying the use of PCA and multispectral measurements.

3.7 Excluded Measurements and Benign Classification Results

Images of 14 of 49 resected ovaries were necessarily excluded from classification. Five were excluded because of extensive external blood on the ovarian surface. Three were excluded because of dye present from another study. Three were excluded because of device malfunction or improper setting. Two were excluded because, although cancerous, the ovaries had been invaded by a cancer that originated elsewhere in the body. Finally, one ovary image set was excluded because tumor growth was so extensive that no ovary remnant could be identified.

Table 2 classification results include only the normal and cancer ovaries, or 26 of the 35 ovaries analyzed. Nine ovaries of a benign neoplasm or endometriosis pathology designation were also imaged. An identical classification of these nine ovaries into normal or cancer classes was performed using the same training data. All 10 benign neoplasm ovary images classified as
normal, whereas the 30 images of endometriosis ovaries were evenly split with 10 normal classifications, nine cancer, and 11 indeterminate.

4 Discussion and Conclusions

We have demonstrated that previously collected single-point autofluorescence measurements of tissue biopsies can be used in combination with newly acquired multispectral images of fresh surgical specimens to effectively diagnose macroscopic areas of tissue. Although single-point fluorescence data were collected in the form of a high-resolution EEM, we transformed them for comparison with eight-point fluorescence spectra captured by the MSI. We created pixel-by-pixel diagnostic maps of the imaged tissue, incorporating the independent, single-point measurements of similar tissues as training data for LDA classification of MSI-measured spectra.

Spectral shape correction was required for effective classification because, although both devices were calibrated for their individual spectral responses and their measurement intensities equalized, the average MSI-collected and probe-collected spectra were different [Fig. 6(a) and 6(b)]. Specifically, the MSI appeared to collect lower relative fluorescence in the violet emission region (especially 420 nm) and to emphasize the longest emission wavelengths (>500 nm). The peak of the average eight-point spectrum was redshifted by one point (from 455 to 485 nm). Redshift of imager-collected spectra relative to probe-collected spectra has been reported by others\(^{43,44}\) and is believed to occur due to differences in the illumination-collection geometries of spectral imager and spectroscopic probe. Gebhart et al.\(^{43}\) determined that the illumination-collection geometry of their imager, on average, led to collected photons with increased tissue interaction compared to those collected by their probe system. We show here that simple shape correction of imaging
spectra for one-to-one comparison with single-point spectra produces good agreement for classification.

Ovary autofluorescence images were divided by normalized, crossed-polarization, green reflectance band images to compensate for spatial variations in the tissue optical properties caused by hypervascular areas and surface blood. The division-by-reflectance method is similar to reported corrections of tissue fluorescence spectra and images. During initial classifications, without this correction, very low fluorescence was measured from reddened tissue areas and resulted in misclassification of those portions of normal ovaries as cancer. Since we required 90% of image ROI pixels to classify correctly for a successful overall classification of the ovary, a number of false positives resulted without the correction, and specificity was 51.1%. Applying the division-by-reflectance correction improved the specificity to 68.5% without changing the sensitivity (100%) and placed classification performance on the level predicted by training set jackknife analysis (100% / 71.5%).

It should be noted that no reflectance measurements existed for the single-point autofluorescence training set, and thus it was not possible to correct the training data in the same manner as the MSI data. We justify classification of the reflectance-modified test set using the unmodified training set by noting that reflectance images were individually normalized (given mean pixel value of one) before the division, maintaining the fluorescence images’ average intensities. Surface blood and hypervascular tissue areas also likely had less influence on the training set data, which were collected by a probe interrogating small (< 1 mm diameter), carefully selected tissue areas. As a result of the normalization of the reflectance images used for correction, tissue optical property spatial variations within particular images were compensated, but image-to-image and ovary-to-ovary tissue optical property variations were not.

Classification of ovarian tissue images based on 365-nm excitation autofluorescence alone does not appear to be sufficient as a technique for diagnosis of ovarian cancer because although sensitivity of the technique was high, specificity was low (51.1%). Blood in the ROI appeared to be the major confounder in about half of the normal ovary images that misclassified. The cause of the other half of misclassifications is unclear but may be due to general variation of tissue optical properties. Correction by green reflectance data led to a more useful specificity of 68.5%. Still higher specificity is desired.

| Table 2 | Classification results using linear discriminant analysis. |
|------------------|------------------|------------------|------------------|------------------|------------------|
| Single-point training spectra | Fluorescence | Fluorescence/green reflectance | Summed fluorescence | 455 nm fluor. only |
| Sensitivity % | 100<sup>a</sup> | 100<sup>b</sup> | 100<sup>b</sup> | 100<sup>b</sup> | 100<sup>b</sup> |
| Specificity % | 71.5 | 51.1 | 68.5 | 38.0 | 31.5 |

<sup>a</sup>Based on 211 single-point measurements; 25 measurements of 10 cancerous ovaries and 186 measurements of 65 normal ovaries (typically three per biopsy).
<sup>b</sup>Based on 104 images of 26 ovaries; 12 images of two cancerous ovaries and 92 images of 24 normal ovaries (typically three to five images per ovary).
and might be obtainable in an imager using more than one excitation wavelength. Spectroscopic studies of the cervix and ovary have demonstrated higher specificities than obtained here, but these incorporated multiple excitation wavelengths to target various endogenous fluorophores. A second alternative to combat the problems associated with blood and tissue optical property variation would be to use FLIM, which is less sensitive to these confounders. Such a system, however, would be more complex, more expensive, and require longer acquisition times.

In this study we have found that benign neoplasm ovaries could be correctly classified as nonmalignant with a normal versus cancer binary classification. This result makes the diagnostic findings on normal and cancer ovaries more meaningful. Endometriosis ovaries, however, are frequently misclassified as malignant in such a binary test. Presumably this result stems from discoloration of the ovary and additional absorption of fluorescence with the presence of additional near-surface vasculature. Our approach of correcting for tissue absorption differences with division by green reflectance data was insufficient to classify endometriosis ovary images as nonmalignant in the binary classification.

All of the measurements used in this study, both single-point and from imaging, have been performed ex vivo. In vivo ovary measurements as well as ex vivo imaging have been reported previously by our research group. A long-term goal of the authors is to integrate this technology into laparoscopy for in vivo diagnostics and real-time cancer screening of individuals at high risk for developing ovarian cancer. Real-time versions of the diagnostic device will not use manual selection of an ROI, but rather classify tissue over a predefined portion of the field of view. Classification maps would be acquired at discretion of the surgeon because of the need to expose tissue to ultraviolet light. If high sensitivity of this technique is confirmed with in vivo studies, the optimal time point of prophylactic oophorectomy could be determined. The described method also would appear to be useful in tumor delineation or selective oophorectomy (only one ovary removed). Additional studies including coordinated sectioning and histopathology would further determine the value of adding multispectral capabilities to endoscopy.

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References

APPENDIX B – RATIO IMAGES AND ULTRAVIOLET C EXCITATION IN AUTOFLUORESCENCE IMAGING OF NEOPLASMS OF THE HUMAN COLON
Ratio images and ultraviolet C excitation in autofluorescence imaging of neoplasms of the human colon

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Ratio images and ultraviolet C excitation in autofluorescence imaging of neoplasms of the human colon

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Abstract. The accepted screening technique for colon cancer is white light endoscopy. While most abnormal growths (lesions) are detected by this method, a significant number are missed during colonoscopy, potentially resulting in advanced disease. Missed lesions are often flat and inconspicuous in color. A prototype ultraviolet spectral imager measuring autofluorescence (AF) and reflectance has been developed and applied in a study of 21 fresh human colon surgical specimens. Six excitation wavelengths from 280 to 440 nm and formulaic ratio imaging were utilized to increase lesion contrast and cause neoplasms to appear bright compared to normal tissue. It was found that in the subset of lesions which were most difficult to visualize in standard color photographs [low contrast lesions, (LCLs)] a ratio image (F340/F440) of AF images excited at 340 and 440 nm produced extraordinary images and was effective in about 70% of these difficult cases. Contrast may be due to increased levels of reduced nicotinamide adenine dinucleotide, increased hemoglobin absorption, and reduced signal from submucosal collagen. A second successful ratio image (R480/R555) combined two reflectance images to produce exceptional images especially in particular LCLs where F340/F440 was ineffective. The newly discovered ratio images can potentially improve detection rate in screening with a novel AF colonoscope. © 2013 Society of Photo-Optical Instrumentation Engineers (SPIE).

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Keywords: endoscopy; fluorescence; medical imaging; multispectral imaging; reflectance; ultraviolet; colon; cancer; gastroenterology.

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1 Introduction

White light colonoscopy is the preferred screening technique for colon cancer but fails to detect a significant number of polyps and flat neoplasms. Improving the detection rate will help prevent incident cancers and decrease screening intervals and thus reduce the overall cost of colon cancer screening. Flat lesions, loosely defined as having a height less than half their width, are of special concern because they are more frequently cancerous than polyoid lesions, and in a recent study, the prevalence of flat lesions was determined to be 9.4% of male veterans undergoing colonoscopy. Indigo carmine chroendooscopy increases the detection of flat neoplasms, but is too time consuming for use during screening exams in the U.S. where a high volume of colonoscopies must be performed under demanding time constraints.

Identifying flat lesions using an endogenous contrast mechanism such as autofluorescence (AF) could provide benefits over chromoendoscopy by reducing exam time and eliminating dye toxicity concerns. AF image contrast in tissue is derived from the native tissue fluorophores [tryptophan, collagen, reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD)] as well as from the effects of absorption and scattering of other components (hemoglobin). Spectroscopic AF studies comparing normal and neoplastic tissues have consistently noted reduced AF intensity from neoplasms. This general result was obtained in colon tissue at excitation wavelengths including 337, 370, and 442 nm. Discrimination based on reduced AF intensity has put emphasis on correcting AF intensity measurements for variations in absorption, illumination intensity, and tissue surface morphology. It has also drawn attention to methods such as time-resolved AF imaging, which largely avoids the confounding factors for intensity measurements but has its own drawbacks including instrumentation complexity and long acquisition times.

The correction of absorption and scattering effects in tissue AF images derives from a mathematical method used to remove such distortions from tissue AF spectra measured with a fiber optic probe. That method relies on collection of both reflectance and AF spectra as well as knowledge of the tissue optical properties. The simplified correction extended to AF imaging does not require knowledge of optical properties and is performed by dividing collected AF intensity at each emission wavelength by collected diffuse reflectance at the same wavelength. Similarly, dividing an AF image by a cross-polarized reflectance image at the excitation wavelength has been shown to produce a ratio image corrected for variation in excitation-collection geometry and irregular tissue surface.
Commercial AF endoscopes for the colon include the AFI system (Olympus Medical Systems, Tokyo, Japan) and PINPOINT system (Novadaq Technologies, Mississauga, Ontario, Canada; formerly the Onco-LIFE and LIFE-GI system, Xillix Technologies). The first developed was LIFE-GI\textsuperscript{15} which illuminated tissue with blue light (400 to 450 nm) and measured both green AF (490 to 560 nm) and red AF (>630 nm). Later generations of LIFE-GI\textsuperscript{15} have included simultaneous blue and red illumination and ceased collection of red AF in favor of red reflectance (>630 nm). The AFI system\textsuperscript{17} illuminates with blue light (395 to 475 nm) and green light (540 to 560 nm) in succession and measures green/orange AF (490 to 625 nm) followed by red reflectance. Both of these commercial endoscopes electronically combine a blue-excited AF image and a reflectance image in a single pseudocolor image presented to the physician. Color differences in the pseudocolor composite image are used to signal the observer to the presence of a lesion. Contrast in images from these endoscopes is produced primarily by a loss of green AF in lesions and is perceived as a changed color ratio. Intensity artifacts due to geometrical shape of the specimen would be apparent in a single image; however, they are reduced in the pseudocolor image because the AF and reflectance images are affected by the same artifacts, preserving the color ratio of the resulting image. Several randomized trials comparing AF endoscopy in the colon to standard video endoscopy, narrow band imaging (NBI), or high resolution endoscopy have been published very recently. The outcomes of these studies have been mixed, with some indicating these AF endoscopes can reduce polyp miss rate\textsuperscript{8–20} and others showing no significant improvement of AF over other technologies.\textsuperscript{21–23}

Other studies have also combined tissue AF images at multiple wavelengths or an AF image and diffuse reflectance image. For example, AF-based ratio images in the near infrared were used to highlight carcinoma in resected breast tissue.\textsuperscript{24} Ratio images in the ultraviolet were found to enhance structure visibility in microscopic images of human esophagus.\textsuperscript{25} A third study used AF ratio images to highlight adenomatous regions in cross sections of excised colon polyps.\textsuperscript{26} Still others have demonstrated the use of AF ratio images microscopically for monitoring cell metabolism and differentiating cancerous tissue.\textsuperscript{27–29}

Previously, we reported early results from an AF imaging study of fresh surgical specimens of human colon.\textsuperscript{30} In this paper we expand on that study and describe in detail the development of a prototype multispectral imager with ultraviolet C (UVC) excitation capability. Analyzing spectral images of cancer and polyps, we experiment with novel ratio images, some employing multiplication, addition, and three or more image types. Using a combination of quantitative and qualitative measures, we identify the most effective ratio images in terms of lesion contrast, predicting that an exceptional contrast in this study will translate to an increased detection of flat lesions in the clinical setting. Finally, we compare our formulated ratio images to commercial AF technology and discuss the potential of this AF method for incorporation in an advanced endoscope for use in colorectal cancer screening.

## 2 Materials and Methods

### 2.1 Instrumentation

The imaging system used to collect both AF and reflectance images was a prototype developed in our laboratory (Fig. 1). The spectral imager with extended UV range (illumination from 260 to 650 nm and detection from 340 to 650 nm) and fixed working distance of 25 cm was used to collect macroscopic tissue images, 4 cm\textsuperscript{2}, at the surface of resected tissues. The light source was a xenon arc lamp system (300 W, Lambda LS, Sutter Instruments, Novato, California) with a built-in ten-position filter wheel. Use of a full-spectrum bulb allowed significant output in the 260 to 300 nm range but necessitated a custom UV cold mirror (Chroma, Bellows Falls, Vermont) with high reflectance over the same range and an ozone filter (Oriel Instruments, Irvine, California). A filter wheel controller (Lambda 10–3, Sutter Instruments, Novato, California) provided automated selection of both illumination and detection wavelengths. A custom quartz fiber bundle (FiberTech Optica, Ontario, Canada) delivered illumination to the specimen and included a feedback fiber for monitoring lamp power fluctuations. The thermostatically cooled, UV-enhanced camera (PhotonMAX.512B, Princeton Instruments, Trenton, New Jersey) with intensified CCD (e2v CCD97B, e2v technologies, Chelmsford, England) was mounted on a rigid mobile arm and equipped with a color-corrected UV imaging lens (f/3.5, f = 63 mm, Resolve Optics, Chesham, United Kingdom). A second ten-position filter wheel was mounted directly in front of the imaging lens. A LabVIEW (National Instruments, Austin, Texas) interface was developed in our laboratory and used to control the two filter wheels, single mechanical shutter, and image acquisition. Automation via scripting allowed sequential capture of 27 different images of interest (and their corresponding dark frames) in about 90 seconds.

All illumination was narrowband (~20 nm full-width-half-maximum) and accomplished with bandpass interference filters, whereas on the imaging side, longpass filters were used exclusively. Longpass emission filters were selected to enable collection of weak AF using shortest exposure times. Illumination for diffuse reflectance images was passed through a removable UV polarizer (UV1 precision linear, Meadowlark Optics, Frederick, Colorado) and collected through a second UV
polarizer mounted in the emission filter wheel. AF contribution to the reflectance images was neglected because it is typically 1000 times less than reflectance.

The excitation wavelengths and emission bands of the AF images were chosen to target native fluorophores (tryptophan, collagen, NADH, FAD, porphyrins) whose concentrations and distributions may change with disease state. Table 1 defines six AF images of interest (F280, F320, F340, F370, F400, and F440), named according to excitation wavelength, and also lists the targeted tissue fluorophores. Excitation and emission spectra of several of these fluorophores are known to overlap, which leads to influence of nontargeted fluorophores in some images. Each of the AF images is formed by subtraction of two different longpass-filtered images. For example, the 320-nm-excited AF image, F320, is formed by subtracting 410-nm longpass image from 375-nm longpass image to create an AF image associated largely with collagen. The six diffuse reflectance images collected are referred to as R370, R400, R415, R440, R480, and R555 where the names specify center wavelength of narrow band illumination. The crossed polarization of these images greatly suppresses specular reflections.

2.2 Instrument Verification

Performance of the imaging system was characterized prior to the clinical study. First, a spectrophotometer (Cary 5, Varian Inc., Palo Alto, California) was used to obtain transmission spectra for all system optical filters. Bandpass excitation filters all possessed out-of-band attenuation four to five orders of magnitude greater than in-band. All the longpass emission filters showed five orders of magnitude greater attenuation below the cut-on wavelengths. Illumination exiting the fiber bundle was measured using a portable spectrophotometer (USB2000, Ocean Optics, Dunedin, Florida). Illumination at 320, 340, and 440 nm revealed no measurable out-of-band light (indicating out-of-band illumination at least 5 orders of magnitude weaker than in-band). Often two filters were stacked to achieve this desired condition. The 280 nm illumination had no measurable out-of-band light besides a spectral feature at 825 nm which was 4.4 orders of magnitude below peak intensity. Though weak, this near infrared light had potential to influence the long pass filtered images of weak fluorescence collected with our camera. Therefore, we additionally placed a shortpass filter (FF01-680/SP, high transmission 345 to 655 nm, Semrock Inc., Rochester, New York) permanently in front of the camera’s imaging lens.

In order to estimate the maximum possible influence of reflected excitation light in tissue AF images, a cuvette of $\text{Al}_2\text{O}_3$ powder (nonfluorescent, high-reflectance material) was imaged side-by-side with a finger of one of the investigators. Ratios were calculated of AF image intensity at the finger to AF image intensity at the $\text{Al}_2\text{O}_3$ powder. For images F280, F320, F340, F400, and F440 the respective ratios were 19.6, 10.1, 13.7, 5.8, and 8.4. Therefore the influence of reflected light leakage in an F280 image is less than 5%, while in F400 its influence may reach 15%. This confirmed that the AF images would be dominated by fluorescence.

2.3 Clinical Measurements

The imaging study was approved by the institutional review board of the University of Arizona Medical Center. Prior informed consent was obtained from patients undergoing colonic resection for clinical indications. Colon specimens were obtained fresh from surgery and imaged thirty to 60 min after excision in a separate darkened room in the same building. Specimens were positioned with the luminal surface facing up and rinsed with saline to remove any blood or stool. Images were composed to include the edge of the lesion allowing comparison with the surrounding normal mucosa in a single photograph. Following spectral imaging, color reference images of specimens were collected using a standard digital SLR camera. Histopathology of each specimen was obtained and used as the gold standard for diagnosis.

Steps were taken to ensure that clinical AF images could be compared quantitatively. During each tissue imaging sequence, optical power meter readings were collected via the feedback branch of the bifurcated optical fiber bundle. Upon completion of specimen imaging, power from the feedback fiber was measured again, and a secondary reading was collected at the specimen imaging location. Solid fluorescence standards (Starls Ltd., Hainault, United Kingdom) including naphthalene, ovalene, tetraphenylbutadiene, and compound 610 were also imaged and reflectance images taken of a flat Spectralon target (Labsphere, North Sutton, New Hampshire).

The study has thus far included imaging of specimens from 30 patients as detailed in Table 2. Twenty-one of 30 specimens from colorectal surgery involved adenocarcinoma (cancer). The low number of adenomas (adenomatous polyps) acquired reflects that adenomas are precursor lesions removed by surgery only when colonoscopic removal is not feasible. To reduce influence of factors not being studied, nine specimens were omitted from the analysis. Factors leading to omission from quantitative AF analysis included highly ulcerative (bloody)

<table>
<thead>
<tr>
<th>Name</th>
<th>Emission (nm)</th>
<th>Target fluorophore</th>
<th>(Other)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F280</td>
<td>345–410</td>
<td>Tryptophan</td>
<td>Pyridoxine</td>
</tr>
<tr>
<td>F320</td>
<td>375–410</td>
<td>Collagen</td>
<td>Pyridoxine</td>
</tr>
<tr>
<td>F340</td>
<td>410–500</td>
<td>NADH</td>
<td>Collagen, Elastin</td>
</tr>
<tr>
<td>F370</td>
<td>410–500</td>
<td>Collagen</td>
<td>NADH, Elastin</td>
</tr>
<tr>
<td>F400</td>
<td>600–655</td>
<td>Porphyrins</td>
<td></td>
</tr>
<tr>
<td>F440</td>
<td>500–600</td>
<td>FAD</td>
<td>Collagen</td>
</tr>
</tbody>
</table>

### Table 2 Study specimens imaged and documentation of exclusions.

<table>
<thead>
<tr>
<th></th>
<th>Specimens</th>
<th>Adenocarcinoma</th>
<th>Adenoma</th>
<th>Other</th>
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<tr>
<td>Overall</td>
<td>30</td>
<td>21</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Final #s</td>
<td>21*</td>
<td>14</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

*Excluded from quantitative analysis were: highly ulcerative lesions (n = 3), tissues from patients with chemo/radiation treatment (n = 3), cancers not originating in colon (n = 1), cases with equipment issues (n = 2)
lesions, lesions that were subjected previously to chemotherapy or radiation treatment, cancers not originating in the colon, and equipment issues.

2.4 Image Processing and Analysis

Raw images were processed using MATLAB (The MathWorks, Natick, Massachusetts). Dark current and room light were compensated by subtracting corresponding dark frames (illumination blocked with shutter). Flat fielding was performed through division by normalized images of a white standard. Source power variation was compensated through division by single correction factors representing relative power readings on date of imaging. Integration times were corrected for as appropriate using a multiplicative factor.

To facilitate quantitative image analysis, regions of interest (ROIs) were selected manually for each specimen with input from a physician experienced in colonoscopy. Circular ROIs were traced over the F280 specimen image in MATLAB while referencing a color reflectance image as illustrated in Fig. 2. This task was performed while blinded to all other image types. A set of one to three ROIs was chosen to represent the lesion and a separate set chosen to represent the most normal appearing tissue. Both ROI sets were saved and later applied in analysis of the AF reflectance, and ratio images. In cases where two or more fields of view of a specimen had been imaged, a single image set involving just one field of view was selected by observing only the color reference images.

2.5 Quantitative Image Analysis

Two different metrics were employed to quantify lesion visibility and evaluate the various ratio and nonratio images. The first metric [Eq. (1)] is based on the well-known Weber contrast which takes the form \( \frac{I_A - I_B}{I_B} \). The second metric [Eq. (2)] was developed and optimized by the authors. In Eq. (1), \( I_{\text{Lesion}} \) and \( I_{\text{Normal}} \) are the mean pixel intensities inside the respective ROIs of a specimen image. Similarly, in Eq. (2), \( I_{\text{Lesion,75\%}} \) and \( I_{\text{Normal,75\%}} \) are the 75th percentile pixel intensities inside respective ROIs of the specimen image. Furthermore, \( \sigma_{L,N} \) is the pooled standard deviation as calculated from the individual standard deviations, \( \sigma_L \) and \( \sigma_N \), of the pixel intensities inside the respective ROIs.

\[
C_{\text{Weber}} = \frac{I_{\text{Lesion}} - I_{\text{Normal}}}{I_{\text{Normal}}} \quad (1)
\]

\[
C_{\text{Opt}} = \frac{I_{\text{Lesion,75\%}} - I_{\text{Normal,75\%}}}{\sigma_{L,N}} \quad (2)
\]

where \( \sigma_{L,N}^2 = \frac{(\sigma_L^2 + \sigma_N^2)}{2} \)

The optimized contrast metric [Eq. (2)] was formulated because the Weber contrast varied ratio images of the form 1/(A * B) over those of forms A/(A + B) and A/B. Ratios of the form 1/(A * B) tended to greatly magnify the numerator of Eq. (1), but also greatly increased the spread of ROI pixel intensities, limiting observed contrast improvement. The optimized metric was also designed to account for the standard deviation, or spread, of the ROI pixel intensities. The 75th percentile intensity was selected as a more consistent measure of ROI intensity in the ratio images (where highly asymmetric distributions of pixel intensities were observed).

To aid in evaluation of the two contrast metrics, authors T.R. and U.U. determined visual contrast scores for each of the formulated ratio images (observing them side by side, one specimen at time). A visual score from one (no useful contrast) to four (best lesion contrast) was assigned to each formulaic ratio image for each of nine specimens in a study subgroup. The most effective contrast metric was taken to be the one with highest correlation to the visual scores of lesion contrast.

2.6 Image Display and Visual Analysis

For selection of the most useful formulaic ratio images, side-by-side visual comparison was effective but heavily dependent on
comparable display of various ratio image types. Division by low pixel values and multiplications produced a wide range of ratio image intensities and a variety of distributions making consistent display a nontrivial task. Following calculation of ratio image intensities, we found it necessary to eliminate the very highest pixel intensities by observing a collective histogram of pixel intensities from all ratio images of the same type and setting all values above a threshold to that threshold level.

Three general methods were used to scale image data for display in grayscale on a monitor. In the "fixed" scaling method (similar to the initial thresholding step applied to all ratio images), the intensities from a large number of images of the same type were observed collectively and suitable minimum and maximum intensity levels chosen to set the low and high ends of the display range. Scaling was "fixed" because every image of the same type was scaled identically. Fixed scaling was appropriate for the AF and reflectance images because the overall variation in intensity values was low. The second method, "autoscaling," is an adaptive method because new minimum and maximum intensity levels are chosen for each image. This ensures every image will have the same amount of completely white and completely black pixels and may alternately be called saturation scaling. The third method, "histogram equalization" is an adaptive method which not only determines appropriate maximum and minimum intensity levels, but also performs the scaling nonlinearly such that the image’s intensity distribution resembles a preselected shape. The result is an image more vibrant but also less quantitative. In scaling each formulaic ratio image for display, either autoscaling or histogram equalization was preferred. As part of all three scaling methods, 1% of intensities were intentionally saturated at both the high and low ends to increase contrast with little loss of information. Scaling methods were applied solely to improve visual appearance of displayed images; all quantitative calculations of contrast were carried out using unscaled image data.

For formal visual analysis, various formulaic ratio images of the same colon specimen were displayed side-by-side in a tiled image. Each ratio image was scaled for display using the preferred method for the specific ratio image type. The identity of each tiled ratio image was concealed from the observers. Visual image scores were individually recorded by seven persons (T.R., B.B., L.G., N.R., U.U., R.L., B.B.) familiar with the project including two physicians (one of these an experienced colonoscopist) and two engineering faculty. Attending researchers were asked to indicate in each ratio image whether the lesion contrast was "adequate" for identification and also to note whether the image could be considered "exceptional." This process was repeated for all specimens.

### 2.7 Formulaic Ratio Images

We formulated over thirty novel ratio images while aiming to maximize lesion visibility and brighten lesions in comparison to surrounding normal tissue. Names were assigned to trial ratio images sequentially (R1, R2, R3, etc.). A select group of the attempted ratio images is presented in Table 3. Many formulas incorporate three or more images as well as a multiplication and/or an addition operation. For the ratios that include an addition operation, all images involved were verified to possess similar average brightness or adjusted by a constant factor before ratio image calculation. As a general rule, AF images expected to display increased lesion intensity (F280, F340) were placed in the numerator of ratios, while AF images expected to display decreased lesion intensity (F320, F440) were placed in the denominator. In some cases, simple mathematical inversion was performed (R10, R20). Ratio images related to our previously published redox ratio \( R \) are R15 and R27.

### Table 3  Select group of trial formulaic ratio images. (F440-red refers to an AF image excited at 440 nm with emission collected from 600 to 655 nm.)

<table>
<thead>
<tr>
<th>R</th>
<th>Formula</th>
<th>R</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>R7</td>
<td>( \frac{1}{F_{440} + F_{320}} )</td>
<td>R24</td>
<td>( \frac{1}{F_{555} + F_{440}} )</td>
</tr>
<tr>
<td>R10</td>
<td>( \frac{1}{F_{440}} )</td>
<td>R25</td>
<td>( \frac{1}{F_{555} + F_{440}} )</td>
</tr>
<tr>
<td>R15</td>
<td>( \frac{F_{340}}{F_{340} + F_{440}} )</td>
<td>R27</td>
<td>( \frac{F_{440}}{F_{440}} )</td>
</tr>
<tr>
<td>R20</td>
<td>( \frac{1}{F_{555}} )</td>
<td>R30</td>
<td>( \frac{R_{480}}{F_{555}} )</td>
</tr>
<tr>
<td>R23</td>
<td>( \frac{F_{440} + R_{440}}{F_{440}} )</td>
<td></td>
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</tr>
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### 2.8 Ratio Image Simulation of Commercial AF Endoscope Images

To compare our formulated ratio images to existing technology, we created ratio images (in grayscale) approximating the color contrasts of two different commercial AF colonoscopes. Ratio image R21 (=F440-red/F440) is modeled after the red/green ratio (ratio of red AF to green AF) described by Zeng et al. as determining color in the LIFE-GI system.\(^\text{15}\) Ratio image R22 (=R555/F440) simulates the G/R ratio (ratio of green AF to green reflectance) described by Aihara et al. as approximating color tone in the AFI system.\(^\text{32}\) Intensity differences seen in grayscale images R21 and R22 estimate the color differences seen on each system's pseudocolor display and used to identify lesions. The Onco-LIFE system images could not be simulated because a red reflectance image is required and was not collected as part of this study.

### 3 Results

The mean lesion contrast of AF and reflectance intensity images from the clinical study is displayed in Fig. 3 with specimens stratified by histology. For all image types, the regions representing the lesions produced lower image intensities than the regions of surrounding normal mucosa. Adenocarcinomas were easier to identify than adenomas in both AF and reflectance images, though it should be noted that the majority of lesions in the adenocarcinoma group were well-established masses with increased vascularity. F440 produced the highest mean contrast in the adenocarcinomas studied yet displayed no statistically significant increase in contrast over image types R400, R415, R555, F320, and F370 (T-test, p > 0.05). In the adenoma group, the advantage of F440 was even less significant with only F280, F340, and F400 judged lower contrast by T-test (p < 0.05). The influence of hemoglobin on narrowband reflectance images is evident in the right side of Fig. 3 where the plotted contrast resembles the absorption spectrum of hemoglobin (primary peaks between 400 and 440 nm and a local maximum near 550 nm).
Because the goal of this study was to develop a useful imaging technique for flat and nearly invisible lesions, we analyzed a subgroup of specimens that were most difficult to identify in the RGB color photographs taken with a standard digital camera. We refer to these as “low contrast lesions,” or LCLs. This subgroup included six adenomas and four adenocarcinomas. Plotting the mean Weber contrast of this subgroup (Fig. 4) reveals an advantage for AF image F440 in identifying LCLs. The increase in contrast of F440 over all the other image types was confirmed by T-test comparisons (p < 0.05).

Even with the highest contrast image type (F440), roughly half of all LCLs could not be well visualized. We also observed interference in this image type from blood and geometrical features (folds) which appear dark and could reduce specificity. Formulaic ratio images were sought to gain high visualization of more of the LCLs. Two contrast metrics proposed to aid in evaluating ratio images were tested by plotting the results of each metric versus visual contrast scores and performing linear fits to the data (not shown). Our optimized contrast metric [Eq. (2)] achieved a higher correlation than the Weber contrast [Eq. (1)], with its linear fit yielding an R-squared of 0.58 as compared to 0.21.

Narrowing the pool of ratio images was achieved by first grouping them based on visual similarity. We identified six such groups as well as four ratios seemingly unique to the others. Equation 2 was then used to calculate lesion contrast for all ratio images of all study specimens. The mean optimized contrast metric score and standard deviation of each ratio image type were compared stratifying adenocarcinoma, adenoma, and low contrast lesion. Formulaic ratio images with a high contrast performance in one or more of these subgroups were chosen for additional visual analysis. Also, effort was made to retain at least one ratio from each of the six groups of similar visual impression. This approach resulted in nine formulaic ratio images with high diagnostic potential (Table 3).

In selecting the best high contrast ratio images, we focused on visual analysis. The appropriate method of scaling each formulaic ratio image was determined by observing visual results on images of all specimens. Examples of the effects of autoscaling and histogram equalization are illustrated in Fig. 5. The better display scaling choice was found to correlate with the mathematical form of each ratio. Ratio images such as R27 and R30, having the form A/B and small ranges of intensities, were displayed effectively with autoscaling. This method was slightly less effective for ratios of the form 1/A and 1/(A + B) but still the preferred choice. For ratio image R7 of the form 1/(A * B), histogram equalization was the preferred scaling method for display. When autoscaled, this ratio image
frequently became dark because a small but substantial percentage of pixel intensities were much higher than any others in the image. For ratio image R15, which has the form A/(A + B), histogram equalization was preferred because autoscaling produced a washed out appearance.

Visual analysis of the selected ratio images (Table 3) was conducted by tiling the nine images of a single specimen on a large display, recording visual scores, and repeating the process for all specimens. Results were stratified by LCL versus nonLCL and are displayed in Fig. 6 as percentages of imaged specimens for which a majority of observers deemed the image “adequate” for lesion identification. As narrowband reflectance imaging was not available for the first six specimens in our study, ratio images R20, R24, R25, and R30 were not available for six out of twenty-one specimens. However, this is accounted for by plotting results as percentages of specimens imaged. The data demonstrate that image R10 (1/F440), regardless of specimen group, was never exceeded in its ability to produce adequate contrast. R23 images were nearly identical to R10 in appearance and visual performance. Ratio R27 (F340/F440) frequently produced exceptional images of LCLs as well as adequate performance on nearly the same percentage of LCLs as R10. R30 (R480/R555) produced many exceptional images in both lesion subgroups but produced fewer adequate images when compared to R10. A second stratification (adenoma versus adenocarcinoma, not shown) produced highly similar results due to the majority of the LCLs being adenomas and majority of nonLCLs being adenocarcinomas. Figure 7 provides examples of R27 ratio images for three specimens classified as low contrast lesions. Remarkable lesion contrast is observed for this fluorescence based ratio. Ratio image R30 was effective especially in the more obvious lesions (nonLCLs) but also produced exceptional contrast for a few of the lesions most difficult to identify with white light. Figure 8 presents color photographs and R30 ratio images for three specimens. Two of these three
Fig. 7 Low contrast lesion examples from resected specimens of three patients. (a) Tubulovillous adenoma, (b) Serrated adenoma, (c) Adenocarcinoma. Grayscale ratio images $R_{27} = F_{340}/F_{440}$ presented in (d)–(f) highlight the lesions which are non-evident in colour photos (a)–(c). Dark regions in the lower right corner of image b and center right of image c are caused by tattoo ink applied prior to specimen excision for marking purpose.

Fig. 8 Three specimens and corresponding $R_{30} = R_{480}/R_{555}$ ratio images in grayscale. (a) Adenocarcinoma, (b) Adenocarcinoma, (c) Tubular adenoma. Specimens a and c were low contrast lesions. The smaller specimen shown in (c) and (f) is masked to show only tissue. The brightest regions of (d) and (e) correspond to lesions. In (f) the brightest regions correspond to inflamed mucosa (left) and tattoo ink (right) used previously to mark the lesion location. In (f) the 6-mm polyp is visible directly below the bright tattoo ink.

(a and c) could be adequately visualized neither by ratio image $R_{27}$ nor by $R_{10}$.

To compare the most successful ratio images to existing AF endoscope technology, a second visual analysis was performed using the same procedure as the first but including ratio images $R_{21}$ simulating the LIFE-GI system and $R_{22}$ simulating the AFI system. To reduce potential bias, the eight persons participating were blinded to the identity of the images being evaluated. Results are presented in Fig. 9. An advantage is seen for $R_{27}$ in percentage of LCLs visualized both exceptionally and adequately. The data also demonstrate that performance can be further improved using $R_{27}$ and $R_{30}$ in parallel due to complementary ability of the two ratio image types. Figure 10 shows four images of the same large flat polyp (tubulovillous adenoma) of a proximal colon specimen. The lesion was difficult to visualize in white light (color photograph). Each ratio image in the figure was autoscaled for display, saturating only small fractions of each image. The ratio images simulating color contrasts of the LIFE-GI and AFI colonoscope systems display considerably less contrast than the newly discovered ratio $R_{27}$. The contrasts calculated with Eq. (2) were $-0.80, 3.17, \text{and } 4.52$, respectively for figure images b, c, and d. Compare these results to the mean contrasts calculated for all 10 LCLs (1.86, 2.18, and 3.30, respectively), and we see
a trend of improvement (51%) using R27. A T-test on this data to determine the significance of the increase in all LCLs yields a \( p \)-value of 0.24 indicating that further data collection is warranted.

4 Discussion and Conclusions

In this study, we created formulaic ratio images which improve lesion contrast by incorporating signals attributed to different fluorophores and also reduce geometrical effects which distort measured AF intensity. We confirmed that the green fluorescence image commercialized as the basis for the LIFE-GI system is effective at producing adequate lesion contrast in most of our samples. However, we found that for viewing low contrast lesions (LCLs), the most useful formulaic ratio image combined an F340 image with an F440 image. At the 340 nm excitation wavelength, both collagen and NADH contribute to colon AF.

A previous study found that in normal colon tissue excited at this wavelength, the AF of NADH was overshadowed by signal from submucosal collagen. However, in adenomatous polyps and adenocarcinomas the influence of NADH on the measured AF signal was pronounced and sometimes exceeded that of collagen. In our study, the F340 image showed increased AF intensity in some of the low contrast lesions (data not shown) leading to improvement of the formulaic ratio image \( R27 = F340/F440 \). Though not experimentally proven, we partially attribute the gains in lesion contrast to increases in lesion NADH fluorescence as captured by the F340 images. This explanation is further supported by the finding that NADH fluorescence increases in neoplastic tissues, whereas collagen AF is known to decrease.

The second image type used in the most successful formulaic ratio image was the AF image F440. The dominant contribution to colon AF signal at 440-nm excitation has been shown to come from collagen in the submucosa rather than mucosal FAD. The pronounced AF decrease observed in adenomas and adenocarcinomas excited at this wavelength has been attributed to both mucosal thickening and increased mucosal blood content.

Our visual analysis of formulaic ratio images confirmed 440 nm as the key excitation wavelength for lesion viewing, as \( R10 = 1/F440 \) produced the greatest percentage of useful images. Our study also yielded a combination of two reflectance wavelengths that is very effective in highlighting many lesions. The ratio image \( R30 = R480/R555 \) incorporates green light which is sensitive to hemoglobin absorption and blue-green light which is relatively insensitive to hemoglobin absorption. In this ratio image R555 provided most of the contrast (see Fig. 4) and R480 served as a reference image correcting effects of geometry. This observation indicates that lesion contrast in R30 is likely driven by hemoglobin concentration. R27 produced exceptional contrast in 50 to 70% of LCLs (the number varied slightly with observer group), whereas ratio image R30 displayed exceptional contrast in about 40% of the LCLs including two that were not well visible in R27. R30 also produced exceptional images in about 40% of nonLCLs and was superior to R27 in that group. The complementary performances of R27 and R30 indicate that while lesion contrast in R30 is enhanced by hemoglobin presence, lesion contrast in R27 may be inhibited by it.

In the process of discovering effective formulaic ratio images, we have also drawn important conclusions about ratio imaging in general. We tested forms of formulaic ratio images involving two, three, and four different image types. Ratios such as \( A/B, 1/(A+B), A/(A+B), C/(A+B), 1/(A*B), C/(A*B), (C*D)/(A*B), \) and \( (C+D)/(A+B) \) were tested as well as simple inversions \( (1/A) \) which produce a
complementary image. Ratio images such as $1/(A* B)$, $1/(A + B)$, $1/A$, and $C/(A + B)$ which had more components in the denominator of the formula than in the numerator produced images of very high contrast when scaled properly for display. However, they also highlighted the darkened features of the AF and reflectance images, indiscriminately emphasizing both lesions and less important features like tissue folds. The formulaic ratio images of form A/B appeared to better compensate for geometrical effects and put emphasis on the behavior of the underlying fluorophores. This is in agreement with the observation of Zeng et al., that a second image may be used in an endoscope as a reference image to mitigate geometrical effects.\textsuperscript{13}

In building the prototype imager, emphasis was given to UV imaging capability and ability to illuminate in the lesser explored 260 to 320 nm wavelength range. Despite previous findings of increased 280-nm-excited AF from cells isolated from colonic adenocarcinoma,\textsuperscript{30} such an increase was not observed here in bulk tissue. Rather, both adenocarcinomas and adenomas excited with 280-nm light displayed slight reductions in AF compared to normal mucosa. As neoplasms are known to be hypervascular, the reductions in observed AF may be due to increased hemoglobin absorption. The F280 images attributed primarily to tryptophan AF were notable for their revelation of the smallest and sharpest surface features due to the minimal penetration depth of the illumination wavelength. Ratio images involving F280 were found less useful for identifying lesions than certain others not including F280.

As part of our analysis we also simulated contrast of two commercial AF colonoscopes and found that R27 produced 51% greater mean lesion contrast, a 40% increase in exceptional images of LCLs, and a 60% increase in adequate images of LCLs (Fig. 9). The simulation ratio image $R_{22} = F_{555}/F_{440}$ representing the AF system from Olympus resulted in relatively less mean contrast because F440 and R555 often produced similar images with one partially canceling the contribution of the other. The lower performance could be due in part to the ex vivo nature of this study which can lead to additional accumulation of blood in the tissue due to trauma induced during surgery.\textsuperscript{8} The simulation ratio image $R_{21} = F_{440red}/F_{440}$ representing the former LIFFE-GI system of Xillix performed less effectively. The limited range of our F440red image (600 to 655 nm instead of 630 to 750 nm) may have limited the accuracy of the simulation ratio.

While it is important to note that the AF of a tissue in vivo and after its resection are different, the changes observed in surgical specimens are minimized by prompt measurement following resection. In a study of colon AF, the NADH signal from resected adenomatous polyps was found to decay by one half every 118 minutes, while signals from collagen and FAD were observed unchanged.\textsuperscript{6} Specimens in the current study were imaged 30 to 60 min after resection which corresponds to a 16% to 30% reduction in NADH AF. AF signals from the extracellular matrix (collagen) and tryptophan are not expected to change, as they are less susceptible to oxidation. Future lesion contrast obtained in vivo with F340/F440 ratio images may improve over observations from this study, assuming that NADH AF decays more quickly in neoplasms (following resection) than in surrounding normal mucosa.

The formulaic ratio imaging method is an extension of existing AF imaging such as implemented in Olympus’ endoscopic AFI system.\textsuperscript{34} The primary differences, which present some endoscope design challenges, are our use of UV illumination and narrowband reflected light images. The 340 nm illumination used by ratio image R27 requires a UV source and UV transparent fiber bundle for light delivery adding some expense to the system, but imaging and coupling optics can be fabricated from an economy glass such as N-BK7 which transmits well down to 340 nm. UV mutagenicity must be considered for in vivo application, and it is therefore imperative that the endoscope possess good collection efficiency to minimize UV exposure. Miniature optics with an N.A. of 0.2 will increase the collection N.A. of the endoscope by a factor of five compared with the study imager. Rapid illumination wavelength switching, <2 ms, can be achieved with filter based commercially available systems employing dual scanning galvanometers. Ratio images must be calculated and scaled for display in real time, likely on a split screen next to white light images.

UV illumination also necessitates the establishment of safety thresholds for exposure which do not currently exist for the colon. Prior to clinical research in humans, exposure safety at 340 nm must be studied in an established transgenic mutation model such as the Big Blue® mouse,\textsuperscript{35} in which mutation assays have been found statistically reliable and recommended for cancer risk assessment.\textsuperscript{36} Alternatively, techniques can be employed such as recently described by researchers at the US Food and Drug Administration, Center for Devices and Radiological Health.\textsuperscript{37} The progenitor or stem cells of the colon, for which UV phototoxicity is of greatest concern, are located at the base of the colonic crypts about 500 μm below the mucosal surface.\textsuperscript{30} These cells are relatively well protected at this depth as the 1/e penetration depth of 340 nm light in normal colon has been measured at just 240 microns.\textsuperscript{8} Mutagenicity concerns are further lessened by the rapid, natural shedding and replacement of epithelial cells in the colon.

The ratio image $R_{27} (= F_{340}/F_{440})$ has some similarity to a recently published ratio image composed as the division of a 365-nm-excited AF image by a 405-nm-excited AF image and used to discriminate adenomatous polytrop regions from normal colon in sectioned tissue specimens.\textsuperscript{26} We expect the ratio image R27 to produce better contrast than a 365/405 AF image when imaging in situ polys because it incorporates contrast from both mucosal NADH (F340) and submucosal collagen (F440). Unlike R27, the 365/405 AF image is reported to have only limited influence from the submucosa which the authors partially attribute to hemoglobin absorption.\textsuperscript{26} In this imaging study of surgical specimens we have obtained results indicating that the capability to detect colon neoplasms could be expanded by including both 340 and 440 nm excitation for AF imaging as well as the reflectance bands 480 and 555 nm. These wavelengths incorporate contrast primarily from NADH, submucosal collagen, and hemoglobin. We found a combination of visual scoring and assessment via an optimized numerical contrast metric to be an appropriate approach in identifying formulaic ratio images with good lesion contrast and desirable histograms for viewing. Our data, although limited by sample size, demonstrate a potential to improve on commercially available AF endoscopes using previously unpublished formulaic ratio images. Expanding our sample pool with more low contrast lesions will help in the development of optimized visualization of difficult to identify lesions. Verification work must be performed in vivo following construction of a novel endoscope.
Acknowledgments
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References
APPENDIX C – AUTOFLUORESCENCE MICROSCOPY WITH SUB-300 NM EXCITATION FOR CELLULAR DIAGNOSTICS

Paper was prepared to submit to Lasers in Surgery and Medicine
Title:
Autofluorescence Microscopy with sub-300 nm Excitation for Cellular Diagnostics

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Abstract
Improved methods are needed for evaluating cells such as those acquired through diagnostic needle biopsy. A novel fluorescence microscope with sub-300 nm excitation has been developed and applied to image cells of human pancreata. The autofluorescence (AF) images are believed to be the first of their kind in this organ and provide unique visualization of protein content associated with tryptophan. Protein levels were modulated in nonsecretory cells with a proteasome inhibitor (Velcade), and 40% increase in intensity was observed in the AF images. Very bright AF was observed in primary exocrine cells and is shown to originate from protein-rich secretory granules. The technique may be used to probe protein synthesis tied to the unchecked growth of cancer cells and result in a rapid and effective cellular imaging diagnostic.

1. Introduction
Fluorescence properties of the amino acid tryptophan are well characterized with absorption peak at 280 nm and emission peak near 340 nm. While it is known that sub-300 nm UV wavelengths excite a significant fluorescence response from cellular
tryptophan, only recently have imaging systems been developed and applied to
investigate this as a contrast mechanism. Aside from being a label-free technique,
fluorescence from tryptophan is promising for cell and/or tissue diagnostics for two
reasons. First, as an amino acid, tryptophan is a component of most cellular proteins. Its
fluorescence provides a means to monitor protein content which may be altered
significantly through the cancer-associated dysregulation of cellular growth processes.
Second, the fluorescent response of tryptophan is orders of magnitude stronger than that
of other native fluorophores such as reduced nicotinamide adenine dinucleotide (NADH)
and flavin adenine dinucleotide (FAD), which can allow faster image acquisition and
reduced ultraviolet exposure.

Motivation for fluorescence imaging of tryptophan has arisen from a number of
promising fluorescence spectroscopy studies at short excitation wavelengths. In one such
study, benign and malignant breast tissue samples were discriminated using 300 nm
excitation and a ratio of the fluorescence emission intensity at 340 nm to that at 440 nm.\(^1\)
The I\(_{340}/I_{440}\) ratio was significantly higher in malignant samples. In a second study, the
same intensity ratio (excited with 310 nm excitation) was found to be increased in
metastatic cell lines of rat and mouse as compared to non-metastatic cell lines.\(^2\) The ratio
was described as a comparison of tryptophan fluorescence to NADH fluorescence. A
similar technique has been applied to oral cancer diagnosis using a combination of two
excitation wavelengths (280 and 330 nm) and corresponding ratios (I\(_{330}/I_{470}\) and
I\(_{380}/I_{460}\) respectively) to achieve a positive predictive value of 92.9%.\(^3\) Furthermore, in
a study of bladder tissue, 280 nm excitation and intensity ratio I\(_{350}/I_{470}\) were used to
distinguish bladder cancer tumors from normal bladder tissue with 97% sensitivity and 93% specificity.\(^4\)

While many spectroscopic studies (with excitation from 280 to 310 nm) utilized ratios of UV fluorescence to blue fluorescence to discriminate cancer, several similar studies have reported differences in the absolute fluorescence intensities observed in the UV region. \textit{In vivo} measurements on nonmelanoma skin cancer yielded higher 295 nm excited fluorescence than surrounding normal skin.\(^5\) \textit{Ex vivo} measurements of both esophageal tissue and isolated cells displayed greater 290 nm excited fluorescence in the cancerous samples than in the normal samples.\(^6\) \textit{Ex vivo} measurements of cells extracted from colon cancer also displayed higher intensity than cells from the surrounding normal tissue.\(^7\) However, tryptophan fluorescence signals excited at short wavelengths have also been reported as reduced in malignant versions of cultured human breast cells,\(^8\) human uterine cells,\(^9\) and murine fibroblasts.\(^10\) Further experiments are necessary to confirm these observations and to elucidate the cellular origin of this UV emission.

Imaging of cellular tryptophan has been slowed by limited availability of UV-capable microscope objectives and sub-300 nm light sources. Some early cellular images of tryptophan fluorescence were captured using a three photon excitation technique and 700 nm light while studying serotonin\(^11\) and also with two photon excitation and OPO tuned to 597nm.\(^12\) UV-capable microscopes using single photon excitation were developed for inspecting protein crystals\(^13\) and for quantitative mapping of cellular protein mass.\(^14\) In perhaps the first cancer related tryptophan imaging study, Mujat et al.\(^15\) compared images of primary and HPV-immortalized human foreskin keratinocytes.
The authors noted an increase in mean fluorescence intensity of the immortalized cells, characteristic bright nucleoli, and a mesh like distribution of signal in the cytoplasm which appeared more diffuse in the immortalized cells. Li et al.\textsuperscript{16} used a two photon technique to image normal and cancerous cervical cell lines. Findings included bright nucleoli and a lower ratio of free NADH to tryptophan fluorescence in the cancerous cell line. Lin et al.\textsuperscript{17} imaged human esophagus biopsies with 266 nm excitation and emission above 400 nm, finding that tryptophan was the dominant fluorophore which yielded good image contrast and bright cell membranes. Notable contrast in thick samples was attributed to the very short penetration depth of 266 nm light. Two photon imaging of tryptophan has also been applied to \textit{in vivo} visualization of white blood cells.\textsuperscript{18}

Several potential applications of short wavelength fluorescence imaging are being investigated, but one promising application is the evaluation of cells obtained by needle biopsy. Fine needle aspiration (FNA) and Pap staining of biopsied cells are commonly used to diagnose masses discovered within the pancreas, breast, and many other organs, but the process is time consuming and not sufficiently reliable. Alternatively, biopsied cells might be imaged with 280 nm excitation for discrimination based on protein content and distribution. Metabolic information can be gained using longer UV wavelengths (340 to 380 nm) to excite NADH and blue wavelengths (430 to 480 nm) to excite FAD. Combined information is expected to provide more reliable diagnosis than single excitation wavelength images.

To investigate the potential for such a diagnostic test, a 280 nm excitation fluorescence microscope was developed and applied to tryptophan imaging of both
cultured and primary cells of the human pancreas. Techniques for imaging both cultured cells and cells freshly isolated from tissue are demonstrated. Unique subcellular visualization enabled by the technique is explored, and tryptophan signals from malignant and nonmalignant cells are quantified. The origin of the signal is investigated in acinar (exocrine) cells of the pancreas with standard staining and contrasted with results from ductal (nonsecretory) cells. The signal origin is further verified using the cancer drug Velcade (bortezomib) to modulate the level of proteins in cultured cells. Cells treated with Velcade, a proteasome inhibitor, experience greatly reduced protein degradation resulting in buildup of proteins in the cytosol. Differences in fluorescence signal between treated and untreated cells are used to estimate the range of cellular tryptophan measurements and determine the sensitivity of the imaging technique to variation in cellular protein levels. The results described provide a framework for the basis of a diagnostic test utilizing deep UV fluorescence microscopy to interrogate cells obtained through tissue needle biopsy.

2. Materials and Methods

2.1 Equipment

The microscope used to image living cells was a standard upright model (BX43, Olympus Corp.) with significant modifications. Autofluorescence (AF) imaging was performed in a transillumination configuration (Figure 1) using excitation wavelength bands centered at 280 nm, 370 nm, and 440 nm. Imaging hardware included a water immersion infinity-corrected UV objective (40X/1.15W UApo/340, Olympus Corp.),
quartz tube lens, and a scientific-grade CCD (16-bit, 512x512 pixels, PhotonMAX, Princeton Instruments) with enhanced UV sensitivity. The objective provided sufficient transmission of UV light within the emission band of tryptophan (30% at 340 nm up to 80% at 380 nm) and good transmission (>80%) from 380 nm to 760 nm. However, its very low transmission of 280 nm light prevented the use of a standard epi-illumination configuration and the capture of deep UV transmission images. The light source has been described previously\textsuperscript{19} and was a full-spectrum xenon arc lamp delivering filtered illumination via a quartz optical fiber bundle. Fluorescence imaging was enabled by emission filters manually inserted in the collimated light path above the microscope objective via a custom slide-in holder. All cells were necessarily imaged on quartz slides possessing high transmission at 280 nm. Cell viability was maintained by heating the stage and objective to 37 °C. Image acquisition was automated with computer control of the CCD, excitation filter wheel, and lamp shutter.

Excitation and emission filters were selected for three fluorophores. Tryptophan imaging used two excitation filters (280/20 bandpass, Semrock; 280/17 bandpass, Chroma) and two emission filters (360/40 bandpass, Chroma; 300LP longpass; Chroma). Imaging at 370 nm illumination was designed for NADH and used three excitation filters (2 x 370/36 bandpass, Semrock; UG11-1mm, Schott) and two emission filters (447/60 bandpass, Semrock; 409LP longpass, Semrock). At 440 nm illumination the target was FAD and included two excitation filters (440/21 bandpass, Omega; 438/24 bandpass, Semrock) and two emission filters (560/70 bandpass, Chroma; 520LP longpass, Chroma).
Several steps were taken to reduce the background signal in fluorescence images. Two or more excitation filters were used at each illumination band to increase out-of-band optical density (OD). Measurements of illumination exiting the fiber bundle indicated minor AF from the optical fiber assembly. For 370 and 440 nm illumination, this light was rejected using an additional bandpass filter placed after the fiber bundle, while for the 280 nm case this AF was negligible. In the transillumination configuration, light passes through the sample and excites some AF in the objective lens. This AF was reduced by limiting the spatial extent of sample illumination with a 700 micron pinhole located immediately below the quartz slide and acting as field aperture. The pinhole was aligned (centered on the field-of-view) prior to cell imaging. Standard glass coverslips were used for imaging instead of quartz because they act as a long pass filter suppressing 280 nm excitation light. While standard coverslips do fluoresce in response to 280 nm, their emission was >380 nm and beyond the region in which we collected tryptophan AF.

2.2 Cell culture

The cell lines imaged included hTERT-HPNE [an immortalized line of human pancreatic ductal cells purchased from ATCC (Manassas, VA)] and MIA PaCa-2 [a tumorigenic epithelial line originating from a human pancreatic tumor.] Each cell line was cultured in a complete growth medium, Dulbecco modified Eagle medium (DMEM) containing Phenol Red, at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Adherent cells were seeded over standard glass coverslips in six-well plates and cultured in DMEM until reaching desired confluency of 50 to 70%.
For experiments including drug treatment, coverslips of cells were prepared as described, and Velcade (bortezomib) was administered to selected wells at a final concentration of 100 nM. Other wells received co-treatment by Velcade (100 nM) and cycloheximide (5 μg/ml), where cycloheximide was added 45 minutes in advance of Velcade. Control wells were left untreated. One or more coverslips were identically prepared for every desired imaging time point (16, 24, or 32 hours).

2.3 Image acquisition
At the desired imaging time point, DMEM was replaced by a pre-warmed medium designed for low background fluorescence which consisted of Hank’s Balanced Salt Solution (HBSS) and 4 mM L-glutamine. Cells undergoing drug treatment were immersed in the same imaging solution but with addition of the treatment drugs at the same concentrations. Cells were allowed a minimum of 20 minutes to acclimate to their new environment, while maintained in a portable incubator at 37 °C without controlled CO₂. Each coverslip of cells was imaged, in turn, after removal from HBSS and placement (cell side downward) over a shallow HBSS-filled well on a quartz slide pre-positioned on the heated stage (Figure 2). Images were acquired in the darkened room with fluorescence images being eight seconds in integration time and transmission images of six or ten milliseconds depending on wavelength. When imaging the weaker AF excited by 370 and 440 nm, two consecutive eight-second fluorescent images were captured and summed.
2.4 Staining

Organelle staining and imaging were performed on live cells immediately following AF imaging of those cells. This was achieved on the microscope stage by delivering solutions to and from a 1 mm deep chamber beneath the cell coverslip using syringes and microtubing. Fluorescent stains for nuclei (Hoechst 33342), mitochondria (MitoTracker Red FM), lysosomes (LysoTracker Green), and endoplasmic reticulum (ER Tracker Green) were purchased from Invitrogen (Eugene, OR) and utilized at respective concentrations of 1 μg/ml, 500 nM, 50 nM, and 1 μM. Three stains were administered simultaneously with incubation time of four minutes followed by twice flushing and filling the chamber with low AF media. ER Tracker and LysoTracker were never used together as they could not be spectrally distinguished.

A quick hematoxylin and eosin (H&E) stain was performed on some suspension cells isolated from tissue. Cells were first fixed in 95% ethanol, concentrated, and then distributed on a slide via the smear technique. Upon drying, the slide was dipped 10x in 95% ethanol, 10x in water, 1 minute in hematoxylin, 10x in water, 10x in 95% ethanol, 1 minute in eosin, 10x in 100% ethanol, and ultimately in xylene until clear.

2.5 Image Processing and AF intensity analysis

An equivalent dark image was captured and subtracted from each AF image for dark correction. AF background was removed by subtracting a control image of a clean coverslip with no cells. Flat field correction of each image was performed through division by a normalized and smoothed image of an identically illuminated fluorescent
tape adhered to a coverslip. Light source intensity was monitored at each exposure by an optical fiber fed back to a power meter and also recorded daily through tracking the intensity of fluorescent tape flat field images. For comparison of fluorescence image intensity recorded on different days, corrective scale factors were derived from the fluorescent tape measurement.

AF intensity level was measured from image to image by first thresholding the 280 nm excited AF image to separate cellular areas from those noncellular. Individual cells were not segmented. Instead intensities from all cellular pixels were summed and normalized by the total number of cellular pixels. The result was an AF intensity per cellular pixel measurement for each field of view captured.

2.6 Pancreatic cell isolation

Whole rat pancreas was acquired fresh through humane sacrifice of Sprague Dawley rats. Viable human pancreas was obtained (with approval) in the form of 3 mm cubes cut from surgical resections related to pancreatic cancer. Both rat and human pancreas were dissociated according to a modified version of a protocol by Amsterdam and Jamieson.\textsuperscript{21} In short, pancreas tissue was minced finely with a blade and bathed in a solution containing collagenase, chymotrypsin, and hyaluronidase, which dissolve the extracellular matrix resulting in small clusters of viable exocrine cells. The detailed protocol is provided as a supplement. Pancreatic cells isolated through the protocol were ultimately concentrated and suspended in HBSS containing 4 mM L-glutamine. For imaging, suspended cells were transferred to a shallow well (0.17 mm deep) on a quartz
slide and topped with a coverslip. These cells settled quickly and were imaged resting on the surface of the quartz slide.

3. Results

Autofluorescence images of multiple cell lines were captured in succession using 280, 370, and 440 nm excitation wavelengths. The hTERT-HPNE (immortalized, pancreatic ductal) cell line was chosen as an example of nontumorigenic, nonmalignant pancreatic cells. Figure 3 shows two rather large hTERT-HPNE cells with a characteristic appearance. Note the dramatic difference in the image (Figure 3A) using sub-300 nm excitation as compared with longer wavelengths (Figures 3B and 3C). Significant AF signal extends to the most remote portions of the cell and is more diffuse than when using the longer excitation wavelengths, but bright punctate areas also exist. Nuclei are easily identified as darker than the surrounding cytoplasm. The nucleoli are notably not visible in Figure 3A.

Characteristic results from the second cell line MIA PaCa-2 (tumorigenic, pancreatic, epithelial) are presented in Figure 4. Morphologically, cells from this line were less flat and less spread out than the hTERT-HPNE cells. Specific differences observed in the 280 nm excited AF images include a nucleolus that is visibly bright in most cells and cytoplasmic AF that appears more diffuse with less punctate fluorescence. In the AF images excited at 370 nm, the cytoplasm has a meshlike or stringy appearance characteristic of mitochondria. At 440 nm excitation the AF takes on a spotted appearance with large cell-to-cell intensity variation. MitoTracker and LysoTracker stains
were performed in effort to explain the observed difference between 370 and 440 nm excitation AF images, whose signal is typically attributed to NADH and FAD (found primarily in the mitochondria). Results show similarity between the 440 nm excited AF and the LysoTracker image. Red, green, and blue color composite images at the right of Figure 4 illustrate the relative localization of all endogenous and exogenous fluorescence signals measured. 280 nm excited AF is shown in gray in the endogenous composite image.

To demonstrate that 280 nm excited AF images measure protein levels in the cells, the drug Velcade was used to modulate protein in two cell lines, and the AF response was recorded. Five experiments were performed, each on a separate day. An AF per cellular pixel value was extracted from each image containing 10 to 30 unique cells. The change in AF per pixel in images of treated cell coverslips, as compared with images of untreated cell coverslips, is plotted versus time in Figure 5. The data demonstrate an AF response to treatment with Velcade that increases with time reaching 30 to 40% above the untreated level. The hTERT-HPNE cells were found to respond faster to treatment than the MIA PaCa-2 cells. The experiment was not extended beyond 36 hours because significant numbers of cells were found to undergo apoptosis after this time.

To show further evidence that the AF response to Velcade is due to protein accumulation, AF response was also measured from cells co-treated with both Velcade and cycloheximide. It was hypothesized that cycloheximide, known to block protein production, would prevent the buildup of protein due to Velcade. The result of the
experiment on hTERT-HPNE cells is depicted in Figure 6. Autofluorescence per cellular pixel is determined using the previously described technique. While cells treated with Velcade experienced a 30% increase in AF after 24 hours, the increase was approximately half (16%) for the co-treated cells.

As a step toward evaluating pancreatic cells from needle biopsy, we demonstrated 280 nm excitation AF imaging of pancreatic cells isolated from tissue. The cells harvested through dissociation of tissue were predominantly exocrine in nature. These acinar cells produce and secrete digestive enzyme precursors in the form of protein granules. Imaging was performed on acinar cells harvested both from Sprague Dawley rats and humans. Representative images are presented in Figure 7. Cells are rounded since they are not attached to the glass, and much less subcellular structure is visible compared to the previous images of cells grown on the coverslips. In the cells from rat, nuclei are not easily identified. Most strikingly, the intense fluorescence originates from the central portion of small clusters of two to three cells. In the image of cells obtained from human tissue, the AF is spread more evenly across entire cells, and the nuclei are frequently visible as dark circular areas. Cell clusters are observed but with a less polar distribution of signal.

The polar AF distribution observed in secretory cells is explained by examining the basic structure and arrangement of cells of this type. As illustrated in Figure 8A, acinar cells have two poles, an apical end packed with secretory granules and a basal end dominated by rough endoplasmic reticulum (ER). The cells join together naturally at their apical poles. Image B of the Figure 8 indicates that bright AF originates from this
apical region (center of the cell clusters). H & E stain (Image C) confirms that this portion of the cells is eosinophilic (staining pink/red) which is consistent with secretory granules. AF images of exocrine pancreatic cells excited at 280 nm appear to be dominated by intense signal from the secretory product.

4. Discussion

This imaging study of pancreatic cells was undertaken especially to characterize the AF due to sub-300 nm excitation so that ultimately a test may be developed for differentiation of normal and cancerous cells. At the 280 nm excitation wavelength and 340 to 380 nm emission band, AF is predominantly from tryptophan, an amino acid building block of many proteins. Tyrosine and phenylalanine also autofluoresce but contribute little to the signal because of their lower quantum yields and quenching through resonant energy transfer. Findings of this study demonstrate that AF excited by sub-300 nm wavelengths can be used to observe changes in protein concentration and distribution. Protein levels may be useful for cell diagnostics because they are intimately related to growth processes in the cell which are dysregulated by cancer.

Distinct differences were observed between tryptophan images of secretory (acinar) and nonsecretory (ductal) cells. Images of secretory cells were dominated by localized intense AF from protein packed secretory granules. This signal often overwhelmed making it difficult to visualize details in the rest of the cell. In contrast, cells that did not contain a secretory product generally could be visualized in greater subcellular detail. Peak intensities were three to five times as high in the secretory cells
as compared with nonsecretory cells. In all cell types, nuclei were darker than cytoplasm which may be due to multiple factors including absence of tryptophan in nuclear histone proteins\textsuperscript{24} and very low quantum yield of DNA.\textsuperscript{23} It was clear from the diffuseness of the images and ubiquitous nature of cellular proteins that the signal cannot be attributed to a single organelle. Amongst the cultured cell lines investigated (all nonsecretory), we observed some variations in 280 nm excited AF images. Aside from differing morphology, the MIA PaCa-2 cells were characterized by visibly bright nucleolus, while the nucleolus in hTERT-HPNE cells was not visible. Published tryptophan AF images of cultured cells seen by the authors have all demonstrated bright nucleoli.\textsuperscript{15, 16} The nucleolus is known to be the site of assembly of ribosomal subunits, and must therefore contain ribosomal proteins which might be observed in the AF images. This could be an area for further study as dysregulated ribosome synthesis has been associated with cancer in humans and at least one drug developed to combat it.\textsuperscript{25}

The 280 nm excited AF per cellular pixel was observed in this study to be higher in the MIA PaCa-2 cell line than in the hTERT-HPNE cell line (data not shown). The relative protein content of the two lines, however, cannot be readily concluded because of morphological differences in the cells. MIA PaCa-2 cells were observed to be thicker and rounder than the flat and broadly spread hTERT-HPNE’s.

One of the core findings of this study was that the protein degradation inhibitor Velcade could be used to increase the measured AF excited at 280 nm. This confirmed that the measurement is sensitive to increased cellular protein level. The AF intensity gain observed was 30 to 40\% given 24 to 36 hours of treatment time. This modest range
in signal may reflect that the amount of protein synthesized in nonsecretory cells over 24 hours is smaller than the initial total protein content in the cells. Cells co-treated by both Velcade and the protein synthesis inhibitor cycloheximide underwent smaller increases in AF, increasing evidence of the signal’s correspondence to protein. In experiments where cells were treated with cycloheximide only, change in the AF response was less pronounced and inconsistent (data not shown). While expected to reduce protein in the cell, cycloheximide treatment often produced no change in AF and sometimes a reduction around 10%. One reason for this might be that the proteasome is down regulated when protein synthesis is suppressed. Collectively, the results of the drug experiments indicate that tryptophan-related cellular protein content might fluctuate by 40% due to change in cellular growth processes.

It appears that a diagnostic imaging test based purely on changes in mean tryptophan AF intensity would be limited by a signal that swings 40%, yet this measurement might be combined with others to improve capability. Additional measurements captured in this study include AF images excited using longer excitation wavelengths (370 nm and 440 nm to assess NADH and FAD respectively). A ratio of NADH AF to tryptophan AF was used by Li et al.\textsuperscript{16} to estimate changes in cell metabolism under the assumption that tryptophan AF (protein content) is slow to change and therefore most useful as a simple reference.

One of the assumptions of this work is that protein levels can be useful to discriminate cells in different growth and disease states. In order to make best use of the tryptophan signal, it may be necessary to determine the signal portion originating from
newly synthesized proteins versus from stable proteins. Or similarly it could be instructive to distinguish tryptophan signal arising from larger protein complexes versus signal arising from smaller protein complexes. It is well known that the immediate environment of tryptophan effects its fluorescence emission. For example, low polarity of surroundings shifts the tryptophan emission peak lower by 10 to 20 nm. Therefore, a technique involving images collected at multiple emission bands may enable selective measurement of newly synthesized proteins and provide better diagnostic information as compared to overall protein content. Likewise, AF polarization anisotropy has been used to separate signal of bound and unbound NADH and might have similar utility for tryptophan AF. Finally, a means of distinguishing large and small protein complexes may be achieved via time-resolved AF images.

Although the focus of this study is on 280 nm excited AF, we also collected AF excited at 370 and 440 nm yielding notable observations. Wavelengths similar to these are often used to monitor NADH and FAD respectively, whose ratio (redox ratio) gives an indication of metabolic status. In this study two cells lines were observed, and comparison to a mitochondrial probe showed good correspondence to the NADH image (see Figure 4). However, the FAD image showed greater resemblance to lysosome probe images. Published spectra of lipofuscin,26 a pigment found in lysosomes, indicate that the FAD image may in fact be dominated by lipofuscin and influenced to a lesser extent by FAD. Combination of these two images may therefore be useful for diagnosis but not be accurate for redox ratio calculation.
This study has demonstrated a novel ultraviolet microscope and techniques for imaging tryptophan and associated protein content in both cultured cells and primary cells dissociated from tissue. The purpose was to develop a diagnostic test applicable to cells from needle biopsy. To the knowledge of the authors, the short wavelength AF images of the study are the first demonstrated in pancreatic cells. Experiments incorporating the proteasome inhibitor Velcade demonstrate that the 280 nm excitation image can sense changes in cellular protein level. Though it has long been known that cellular growth processes are dysregulated in cancer, more study is needed to determine how effectively protein level might be used to differentiate normal and transformed cells. Because a large number of factors must be controlled for a repeatable absolute AF measurement, it is more likely that the ultimate solution will be a relative measurement. It may be possible to extract AF information on newly synthesized protein, a parameter which could vary more greatly than an overall protein measurement. Future studies will further investigate metrics of the novel tryptophan images most effective for cellular diagnosis.
Acknowledgements

The authors would like to recognize the contributions of Dr. Ronald Lynch in form of many helpful observations and suggestions for experiments. Funding for this work was provided by the Marshall Foundation, the ARCS foundation, and the University of Arizona Department of Medicine.

References


Figure 1. Deep UV autofluorescence microscope in upright transillumination configuration. Light path is from bottom to top of diagram. Optical elements have high transmission in the sub-300 nm range of interest except for the UV objective which has very low transmission below 340 nm.
Figure 2. Arrangement for cell imaging in the transillumination upright microscope. Cultured cells are attached to an inverted coverslip and immersed in low AF media (HBSS & L-glutamine). Stage and objective heaters (not shown) maintain temperature within 2 degrees of 37 °C. Suspension cells are imaged using the same setup but settle quickly and rest on the top surface of quartz slide.
Figure 3. Autofluorescence images of hTERT-HPNE cells excited at A) 280 nm, B) 370 nm, and C) 440 nm. Note in A the ability to clearly visualize the entire cells and the differences between B and C, most significantly at the region designated by arrow.
Figure 4. Autofluorescence images of MIA PaCa-2 cells excited at A) 280 nm, B) 370 nm, and C) 440 nm and fluorescent probe images D-F taken following 4 minutes incubation of same cells in a multicolor stain. Nuclei are visible as darker regions of cells in both A and B. Note bright nucleoli in image A, two of which are designated with arrow. Image B shows a stringy or meshlike pattern characteristic of mitochondria and corresponds well with the MitoTracker stain in image E. Fluorescent probe images reflect the detachment of a few cells (3 o’clock, 8 o’clock positions) during media exchange. Note the similar appearance of image C to the LysoTracker stain in image F. Color composite images were generated of two cells present in the upper left quadrant of each image. These reveal the relative localization of the endogenous and exogenous fluorophores.
Figure 5. Change in 280 nm excited autofluorescence (AF) for two different cell lines following administration of Velcade. Each marker represents average change in autofluorescence of a Velcade-treated cell coverslip relative to an equivalent untreated cell coverslip. One measurement from each imaged field-of-view (FOV) was obtained by thresholding to locate cellular pixels, summing the AF intensities, and dividing by the number of cellular pixels. Error bars indicate measurement variation according to different FOV’s measured on a single coverslip of cells. Linear fits to the data from each cell line are indicated as trend lines.
Figure 6. 280 nm excited autofluorescence per pixel of untreated, Velcade-treated, and co-treated coverslips of hTERT-HPNE cells measured at 24 hours after drug treatment. The Velcade-treated measurement was repeated for two coverslips and the co-treated measurement repeated for three coverslips. For each coverslip, four fields of view were measured. Velcade, which leads to protein accumulation, resulted in 30% increase in AF with a significance of p=3x10^{-7}. Co-treatment with Velcade and cycloheximide resulted in only a 16% increase with significance p=1x10^{-4}. The difference between Velcade-treated and co-treated cell AF was significant with p=3x10^{-5}.
Figure 7. Autofluorescence (AF) of primary pancreatic cells excited with 280 nm illumination. The exocrine cells imaged were isolated from normal rat and human pancreas respectively. In the sample from rat, clusters of two and three cells display a highly polar distribution of AF, but little subcellular structure is visible. In the human sample, the AF is spread more evenly across the cells, and nuclei are frequently visible as dark circles (as indicated by arrows).
Figure 8. Structure of exocrine pancreas explains observed autofluorescence. A) Sketch of an acinus, illustrating the arrangement of secretory (acinar) cells around a duct and the characteristic H & E staining pattern, B) 280 nm excited autofluorescence of characteristic clustered acinar cells dissociated from rat pancreas, C) H & E stain of representative acinar cells dissociated from rat pancreas. Areas of bright fluorescence in image B correspond to cell regions with a high concentration of protein granules.
The specific filters used in the prototype multispectral imager system are detailed in the tables below and their placement in ten-position filter wheel noted. In many cases two or more excitation filters were stacked to reduce out-of-band illumination from the broadband xenon source. Excitation filters were 25 mm in diameter. Emission filters were 32 mm in diameter.

Table 1. Excitation Filters

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The Cary 5 spectrophotometer was used to collect transmission scans of all excitation and emission filters used in the prototype multispectral tissue imager. In cases where two or more filters were to be stacked in the device, one scan was collected for each stack of filters.

![Figure 1. Excitation Filter Transmission (Log plot)](image-url)
Figure 2. Excitation Filter Transmission (Log plot)

Figure 3. Emission Filter Transmission (Log plot)
Figure 4. Emission Filter Transmission (Standard Plot)

Spectral properties of the illumination from the xenon short arc lamp source were measured using a USB2000 portable spectrophotometer from Ocean Optics (with high-OH fiber optic input). The range of the USB2000 was from 200 nm to 875 nm although the xenon source is known to produce longer NIR wavelengths as well.
Figure 5. Calibrated Spectral Output of source measured before and after passage through fiber optic bundle. Output above 650 nm reduced due to UV cold mirror. Measurement taken without any excitation filter.

For the spectra measured in figures 6, 7, 8, and 9, a technique was employed to extend the dynamic range of the USB2000 measurement by two orders of magnitude. The initial measurement was captured using a short integration time. A following measurement was taken with an additional filter used to block the excitation band and the integration time increased by a factor of 100. Background (BG) spectra were also measured and subtracted to compensate low levels of room light. The measurement taken using blocking filter can be used to estimate the out-of-band performance of the excitation filters. Therefore in Figure 6 the peak intensity of the magenta curve (at 280 nm) can be compared with the peak intensity of the green curve (at 825 nm) showing the out-of-band intensity to be 4.4 orders of magnitude below peak. Similarly, figures 7 and 8 show at
least 5 orders of magnitude reduction in out-of-band intensity. Figure 9 shows approximately 4.2 orders of magnitude reduction.

Figure 6. Calibrated Spectral Output of source using 280-nm excitation filters. (Calibration likely inaccurate below 250 nm.) Note measurable leakage at 825 nm. Note measurement taken prior to optical fiber bundle.

Figure 7. Calibrated Spectral Output of source using 320-nm excitation filters. (Calibration likely inaccurate below 250 nm.) Note measurement taken prior to optical fiber bundle.
It was seen that very little out-of-band light reaches the specimen; however, 
autofluorescence can be 4 to 5 orders of magnitude weaker than reflected light. The
measurements just shown led to the conclusion that it is necessary to place a shortpass filter in front of the camera to block NIR light from possibly interfering with autofluorescence images. The most appropriate filter that could be located was the 680 shortpass from Semrock, Inc. The manufacturer scan of such a filter is plotted in Figure 10. It was necessary to use this filter on the emission side of the system because this filter does not transmit all system excitation wavelengths.

![Figure 10. Typical OD spectrum for FF01-680/SP-25 shortpass filter from Semrock, Inc.](image)
APPENDIX E – RATIO IMAGE EVALUATION, COMPREHENSIVE RESULTS

Table 1. Formulaic ratio images investigated

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<td>R13</td>
<td>( \frac{F_{555}}{F_{280}} )</td>
</tr>
<tr>
<td>R14</td>
<td>( \frac{1}{F_{400}} )</td>
</tr>
<tr>
<td>R15</td>
<td>( \frac{F_{340} + F_{440}}{F_{440}} )</td>
</tr>
<tr>
<td>R16</td>
<td>( \frac{F_{415}}{F_{480}} )</td>
</tr>
<tr>
<td>R17</td>
<td>( \frac{F_{440}}{F_{440}} )</td>
</tr>
<tr>
<td>R18</td>
<td>( \frac{1}{F_{440} \ast F_{400}} )</td>
</tr>
<tr>
<td>R19</td>
<td>( \frac{R_{370} \ast R_{480}}{F_{440}} )</td>
</tr>
<tr>
<td>R20</td>
<td>( \frac{1}{F_{555}} )</td>
</tr>
<tr>
<td>R21</td>
<td>( \frac{F_{440} \ast F_{555}}{F_{555}} )</td>
</tr>
<tr>
<td>R22</td>
<td>( \frac{F_{440}}{F_{340}} )</td>
</tr>
<tr>
<td>R23</td>
<td>( \frac{1}{F_{440} \ast F_{440}} )</td>
</tr>
<tr>
<td>R24</td>
<td>( \frac{F_{555} \ast F_{440}}{F_{555}} )</td>
</tr>
<tr>
<td>R25</td>
<td>( \frac{1}{F_{440} \ast F_{440}} )</td>
</tr>
<tr>
<td>R26</td>
<td>( \frac{(F_{440})^2}{F_{340}} )</td>
</tr>
<tr>
<td>R27</td>
<td>( \frac{F_{440}}{F_{440}} )</td>
</tr>
<tr>
<td>R28</td>
<td>( \frac{F_{440} \ast F_{340}uv}{F_{320}blue} )</td>
</tr>
<tr>
<td>R29</td>
<td>( \frac{F_{320}}{R_{480}} )</td>
</tr>
<tr>
<td>R30</td>
<td>( \frac{1}{F_{555}} )</td>
</tr>
</tbody>
</table>

Figure 1. Contrast in ratio images of cancer (ratios grouped by image similarity)
Figure 2. Contrast in ratio images of cancer, broken down by distal or proximal lesion location

Figure 3. Contrast in ratio images of polyps, broken down by sessile or pedunculated polyp
Figure 4. Contrast in ratio images of lesions of the proximal colon, broken down by cancer or adenoma status of lesion.

Figure 5. Contrast in ratio images of low contrast lesions. Identical markers indicate image of same specimen.

The results of visual analysis of the individual formulaic ratio images (Figure 6, Appendix B) indicated that some improvement could be realized by combining the ability of multiple ratios. Our goal was to identify a subset of two to three formulaic ratio images such that at least one member of the subset would produce high lesion contrast for all of the specimens imaged (LCLs and normal contrast). Using the same criteria as to determine if specimen images were “adequate” and “exceptional”, we established the
most useful combinations of any two of the nine ratios visually assessed. The results are shown in a stacked bar chart, Figure 6, such that the unique contribution of each ratio image can be seen as well as the overlap. The combination of R10 and R30 allowed adequate contrast for the most specimens (20 of 21). Eight other combinations of two ratios achieved 19 of 21; however the three combinations shown are those that would achieve 20 of 21 if voting threshold were lowered from 4 of 7 to 3 of 7. The R30 and R27 combination was included because it results in the highest number of exceptional images. Additionally, the R30 ratio was not available for the first six specimens and potentially could have achieved a score as high as 19 of 21 if it had been available.

Figure 6. Visually-assessed combined performance of pairs of formulated ratio images. Overall bar height represents the percentage of specimen images for which at least one of the two listed ratio images was deemed “adequate” for lesion identification by a majority of observers (at least 4 of 7). The relative size of shaded bar segments indicates the unique contribution of each ratio image, as well as the
contribution that is common to both ratios. Diamonds represent the percentage of specimen images for which at least one of two ratio images was deemed “exceptional” by 3 or more of 7 observers. R10, R30, and R27 were the most important ratio images, and R10 and R30 made the most effective pair for this dataset.

Figure 7. Comparison of lesion contrast in key ratio images. R21=F440red/F440 represents an approximation to the color contrast in LIFE-GI images. R22=R555/F440 represents an approximation to the color contrast in Olympus AFI endoscopic images. R27=F340/F440 and R30=R480/R555 are ratio images finding success in this study. Results have been grouped by status of the imaged lesions as low contrast lesions (LCL) which are difficult to identify in standard viewing under white light. R27 displays a 51% increase in contrast over R22 (p=0.236) for viewing low contrast lesions. R30 shows an 87% increase in contrast over R22 (p=0.039) for viewing non-LCLs.
APPENDIX F – DISSOCIATION PROTOCOL FOR PANCREAS TISSUE

Pancreas Dissociation Protocol modified from Amsterdam & Jamieson 1974*

B: Dulbecco’s Modified Eagle Medium (DMEM/F12) + 0.1 mg/ml soybean trypsin inhibitor (STI)
C: Solution B + 200 U/ml collagenase + 0.2 mg/ml chymotrypsin + 2 mg/ml hyaluronidase
D: Krebs-Henseleit buffer + 2 mM EDTA + 0.1 mg/ml soybean trypsin inhibitor (STI)
E: Solution B + 4% bovine serum albumin (BSA)

--steps 3 to 6 carried out in 37°C water bath shaking at 130 oscillations per minute

1. Collect pancreas in **solution B**
2. Mince desired pancreas portion finely with surgical blade
3. Incubate in **solution C** for 12 minutes at 37C in 25 mL Erlenmeyer flask
4. Incubate in **solution D** for 10 minutes at 37C
5. Incubate in **solution B** for 3 to 5 minutes at 37C
6. Incubate in **solution C** for 40 to 50 min at 37C
7. Pipette up and down 5x using tip diameters of 1.3mm (10mL tube) and 0.9 mm (1 mL pipette tip) at room temp in **solution C**
7b. Remove any undissociated tissue or cell clumps by filtering first through 100 um and then 40 um mesh nylon gauze
8. Layer equal halves of the suspension over duplicate 8 mL cushions of **solution E** at room temp
8b. Centrifuge at 50 g (520 RPM on Jouan centrifuge) for 5 minutes
8c. Wash pellets by resuspension and recentrifugation in 8 mL of **Optically Inert Media (HBSS + L-glutamine)**
9. Resuspend cells in **Optically Inert Media (HBSS + L-glutamine)** at 37C