Fluorescein as a topical fluorescent contrast agent for quantitative microendoscopic inspection of colorectal epithelium

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Abstract: Fiber bundle microendoscopic imaging of colorectal tissue has shown promising results, for both qualitative and quantitative analysis. A quantitative image quality control and image feature extraction algorithm was previously designed for quantitative image feature analysis of proflavine-stained ex vivo colorectal tissue. We investigated fluorescein as an alternative topical stain. Images of ex vivo porcine, caprine, and human colorectal tissue were used to compare microendoscopic images of tissue topically stained with fluorescein and proflavine solutions. Fluorescein was shown to be comparable for automated crypt detection, with an average crypt detection sensitivity exceeding 90% using a combination of three contrast limit pairs.

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1. Introduction

Microendoscopy is an emerging method which can provide clinicians with in vivo information on tissue microstructure that is not readily visible using traditional colonoscopy and endoscopy. It allows for histology-level detail to be acquired in real-time, with the potential for in vivo guidance for the clinician, such as in targeted biopsies [1,2]. Myriad microendoscopic imaging modalities have been applied to various tissues, including ovarian, esophageal, bladder, colonic, and oral epithelium [2–7]. We have chosen to investigate colorectal cancer, as it is currently the second leading cause of cancer deaths in the United States [8]. The precursor of colorectal malignancy is dysplasia and there is a long lag phase between the onset of dysplasia, and the development of malignancy [9]. Detecting these regions prior to becoming clinically evident will further help in preventing colorectal cancer [10]. In addition, patients with inflammatory bowel disease (IBD), who are frequently screened for dysplastic regions, would likely also benefit from early detection of suspicious lesions [11]. Several microendoscopy imaging studies are aimed at improving early detection of glandular epithelial dysplasia, and providing biopsy guidance and prognostic indicators in clinical settings [2, 12–14].

Currently described microendoscopic methods for in vivo imaging of gastrointestinal epithelium vary, including integration of the probe into conventional endoscopes [12], fiber bundle confocal systems for increased depth penetration [1], and portable wide field end-on fiber bundle approaches such as a previously developed system [4]. End-on fiber bundle systems, such as the one used in this manuscript, are designed to come into direct contact with the tissue, and image acquired is either of the tissue directly in contact with the distal end of the probe (for wide field microendoscopy systems), or of varying depths below the epithelium (depth-sectioning with point-scanning systems). Benefits of end-on fiber bundle systems include eliminating the need for rinsing tissue after stain application to remove excess dye, which is required for alternative imaging modalities such as chromoendoscopy [15]. Eliminating the rinsing step is possible since end-on contact reduces specular reflection or unwanted fluorescence, with the additional benefit of rapid imaging after staining since there is no wait time for sufficient incubation to occur before rinsing. While a wide field approach does not have the depth-sectioning capabilities of a point-scanning system, it does not require a pinhole, laser light source and alignment, nor raster-scanning illumination.

Imaging typically depends on contrast between image regions, be it differences in color or in light intensity that highlight features of interest, and contrast can come from various sources, both endogenous and exogenous. Endogenous sources in endoscopic systems include hemoglobin absorption and inherently fluorescent molecules such as NADH and FAD, two prominent markers of metabolism. Exogenous sources of contrast are more commonly added prior to image acquisition, such as chromoendoscopy or fluorescent microendoscopy. Chromoendoscopy depends on the colorimetric contrast provided by stains such as Lugol's iodine or methylene blue [15]. These dyes are typically absorbed by the tissue in varying degrees depending on properties such as increased or decreased cell cytoplasm or goblet cell presence [15]. After these dyes are applied, they then require multiple rinses before imaging under white light endoscopy [16]. A benefit of exogenous fluorescent dye based microendoscopy is the increased signal to noise ratio, as the fluorescently stained regions are emphasized. Examples include fluorescence microendoscopy using acridine dyes [6] and surface magnifying endoscopy using the fluorescent emission of methylene blue [17]. Fluorescently stained regions will vary depending on the dye, for example acridine-derived dyes such as proflavine will preferentially stain nucleic structures, whereas methylene blue will be preferentially absorbed by mucosal tissue [4,15].

Proflavine has been used as a topically delivered, exogenous contrast agent with fluorescent microendoscopy systems due to its DNA intercalating nature. Therefore, proflavine predominantly highlights cellular nuclei in cytology specimens [18], as well as squamous bulk tissue such as oral and esophageal [4,7]. Proflavine is an amphipathic dye...
comprised of a three carbon ring structure, and capable of intercalation within the minor
groove of the DNA helix [19]. Broad acceptance of proflavine and its use in vivo in human
subjects has been limited in the United States as DNA-intercalating dyes are considered
potentially hazardous and mutagenic [20,21]. Fluorescein, on the other hand, has a history of
clinical use as an intravenous contrast agent in retinal angiography, as it does not intercalate
DNA and tends to diffuse between cells rather than permeating them [22]. In addition,
confocal microendoscopy modalities, such as Mauna Kea, have successfully used intravenous
(IV) delivery of fluorescein for gastrointestinal imaging [23]. A limitation of fluorescein is its
use as a contrast agent in squamous bulk tissue, such as oral epithelium, where nuclei shape
and homogeneity is a main indicator of dysplasia [7]. On the other hand, fluorescein has been
shown to have a sensitivity of 88% for detection of high-grade intraepithelial neoplasia in
Barrett’s esophagus, when using IV delivery in conjunction with confocal microendoscopy
[24]. When imaging with wide field modalities, such as the microendoscopy system studied in
this manuscript, we have shown that topical application of fluorescein is sufficient to
highlight crypt structures, while topical delivery via an auxiliary endoscopy probe channel
might minimize the amounts of systemic contrast agent [25].

Adequate contrast agents not only provide the clinician with in vivo qualitative insight, but
can be used to acquire and analyze digital images. Pathology studies of biopsies or sections
typically depend on H&E slides qualitatively analyzed for pre-neoplastic markers such as
nuclei size, shape, distribution, as well as crypt structure [26]. Qualitative analysis of images
acquired with fluorescence microendoscopy by trained observers has been shown to be highly
sensitive and specific [14]. Intraobserver agreement was high, and provided positive results
for subjective review of microendoscopic images [14]. While there is a benefit to subjective
and complementary image analysis, introducing a new imaging modality into an
intraoperative setting necessitates a certain burden of training, namely additional training for
the clinician. Image acquisition can be combined with computer-based analysis and diagnostic
algorithms to provide complementary data to guide clinicians in their analysis of the tissue at
the site of interest [6,27,28]. Automated image analysis provides an objective, replicable
output to lessen the burden of training for clinicians. Our previous work has shown that the
automated detection sensitivity of crypt structures in images of proflavine-stained colorectal
epithelium using a custom algorithm ranges from 71 to 94% [29]. In addition, preliminary
quantification of crypt area and circularity suggest that crypt quantification may provide an
unbiased metric for aiding clinicians using fluorescence microendoscopy systems [26,30].

In this study, we have evaluated the ability of topical proflavine and fluorescein to provide
contrast in colorectal epithelium using end-on fiber bundle microendoscopy imaging of bulk
ex vivo tissue. For qualitative comparison of topical staining, porcine and caprine bulk
colorectal tissue was stained with proflavine and fluorescein solutions and imaged. For
quantitative comparison, we have calculated the automated average crypt detection sensitivity
of our custom algorithm using porcine bulk tissue stained topically with proflavine and
fluorescein. In addition, ex vivo human colorectal tissue was imaged to quantitatively compare
crypt area and circularity in grossly normal and grossly dysplastic regions. These quantified
results provided us with values in order to compare the viability of both these topical contrast
agents for use in conjunction with fluorescence microendoscopy systems. Our results present
topical application of fluorescein as a reasonable alternative to proflavine for fluorescence
imaging of the colorectal tract.

2. Materials and methods

2.1 Fiber bundle microendoscopy system and topical contrast agents

The microendoscopy system has been previously described [6,29], and consists of a 455nm
LED (Philips, USA), a filter set (Chroma Tech, USA), monochrome camera (Flea 3, USB 3.0,
Point Grey Research Inc., CA), a 10x objective (NA 0.25, Olympus, Japan), and a fiber
bundle image guide comprised of 50,000 fibers with 3.5µm center to center spacing (FIGH-
50-1100N fiber, Myriad Fiber Imaging Tech, Inc., USA) with customized ends. The distal end has a modified SMA connector with rounded edges, to reduce the risk of tissue tearing as well as protect the fiber from chipping.

Proflavine hemisulfate was prepared at a concentration of 0.01% (w/v) in 1x phosphate buffered saline (PBS) [29]. Fluorescein salt solution was prepared at 0.05% (w/v) in 1x PBS. We used fluorescein sodium salt, with a reported solubility of 0.1% (w/v) in water (F6377, Sigma-Aldrich), therefore our study investigated at and below this concentration.

2.2 Image intensity as a measure of topical contrast agent concentration

Previous studies of clinical microendoscopy using topical proflavine have used a standard concentration of 0.01% (w/v); we compared three concentrations of proflavine against fluorescein salt solutions with equal weight-per-volumes. We sectioned 6 adjacent regions of porcine colorectal tissue, and stained each section with one of the following contrast agents and concentrations: 0.01% (w/v) proflavine, 0.05% (w/v) proflavine, 0.1% (w/v) proflavine, 0.01% (w/v) fluorescein, 0.05% (w/v) fluorescein, 0.1% (w/v) fluorescein, all solutions made with 1x PBS. Ten images were acquired of each section, with identical camera settings; 50ms exposure and 5dB gain. We qualitatively inspected them, as well as quantitatively compared them by measuring the average image intensity. The average image intensity (N = 10 images) of each solution was plotted; error bars denote standard error [Fig. 5]. Statistical values were obtained from post-hoc testing of a one-way ANOVA using Tukey’s honestly significant difference (HSD), with p-value <0.05 as the criterion for significant difference.

2.3 Animal tissue procurement

Microendoscopic images of freshly resected caprine colorectal tissue were acquired in collaboration with the University of Arkansas Animal Science Abattoir, which provided tissue from recently slaughtered animals at their facility. 12-inch distal colon specimens were resected within an hour of slaughter, and imaged within an hour of resection. Siloam Spring Processing provided porcine colorectal tissue from recently slaughtered animals at their facility. 12 to 24-inch distal colon specimens were resected within an hour of slaughter, and refrigerated for approximately an hour before imaging, and imaged within an hour of removal from refrigeration. Specimens were obtained from two goats and two pigs for the quantitative study in this manuscript. Colon sections were rinsed with water, longitudinally cut to expose the lumen, and the lumen was rinsed with cold PBS. For each specimen, a single region of approximately 2 inches by 8 inches was sectioned for imaging.

2.4 Human tissue procurement

Microendoscopic images of freshly resected human colorectal tissue were acquired in collaboration with the University of Arkansas for Medical Sciences, from consenting patients under IRB protocol (#204651). Consenting patients, over the age of 18, with benign or carcinogenic disease, were accepted under this protocol. Colorectal tissue was resected and sent to the pathology lab, where residents rinsed, longitudinally sectioned, and pinned the colorectal tissue to a paraffin block. Three specimens (two cases of benign disease and one invasive adenocarcinoma) were used for the data in this manuscript, with “normal” images acquired from grossly appearing normal mucosa from each of the three specimens, as well as images of neoplastic mucosa regions in the one specimen with invasive adenocarcinoma.

2.5 Microendoscopy image acquisition

The animal colorectal tissue was first longitudinally sectioned and then placed epithelium side up on a 150mm Petri dish. Colon sections of each specimen, porcine and caprine, were divided using a scalpel, with one section stained with proflavine and the adjacent section stained with fluorescein. No specimen received both contrast agents on the same tissue area. Separate sections of the specimen were used for multiphoton microscopy, though this separate
Human colorectal tissue was pinned to a paraffin block, lumen side up, and for each specimen, two regions of normal colon were chosen, as well as two regions of dysplastic colon. Microendoscopy images were acquired immediately following topical application of fluorescein salt solution (0.05% (w/v) in 1x PBS) with a cotton swab, with occasional re-application of the dye as needed. Individual images were acquired by manually placing the distal end of the fiber bundle in contact with the epithelial surface. Image acquisition lasted approximately 30-40 minutes, with 50ms exposure and 5dB gain settings.

### 2.6 Multiphoton image acquisition for qualitative comparison

Two 1cm² sections of caprine colorectal tissue were placed on individual glass slides, topically stained with either proflavine (0.01% (w/v)) or fluorescein (0.05% (w/v)) solution, and covered with No.1 coverslips, and immediately imaged using a MaiTai (SpectraPhysics) Ti:Sapphire pulsed laser, coupled to a customized multiphoton microscope system with an emission bandpass filter of 525nm center wavelength and 45nm bandwidth (Thorlabs). Images from tissues stained with these two agents were acquired using 900nm two-photon excitation at 5mW power at the sample, and a 40x (0.8 NA) water immersion objective (Nikon). Images comprised a 261µm by 261µm field-of-view, at 512 by 512 pixels.

### 2.7 Crypt detection and quantification algorithm and optimization of parameters

We have previously developed a custom image feature quantification algorithm (QFEA) for automated crypt detection and quantification of crypt morphology in microendoscopic images of proflavine-stained colorectal tissue [29]. This algorithm was designed to detect, segment, and quantify area and circularity of crypt shapes in microendoscopic images, and calculated automated crypt detection sensitivity at the crypt level by comparing if the algorithm detected a crypt at the position that the trained observer visually detected a crypt [29]. We have optimized this algorithm for images of fluorescein-stained colorectal tissue, and compared the crypt detection sensitivity of the algorithm for proflavine versus fluorescein-stained epithelium, as described below. Prior to image quantification motion-blurred images were excluded from quantification, then the signal-to-background ratio was calculated, which we have termed the Quality Ratio (QR). We designed the QR metric to be a crypt-specific signal-to-background ratio [29], as the pixel-by-pixel signal-to-background ratio can vary and be misleading when calculated. The QR metric smooths individual pixel peaks and troughs, in order to calculate the more general signal-to-background ratio of the crypts [6]. Pixel intensity line profiles were extracted from the image in a grid like pattern, and the peak-to-trough ratios of the profiles were averaged to calculate the image’s QR [Fig. 1(a)] [29]. Figure 1(a) shows an example of the line profiles crossing through a crypt, with a representative example of a line profile through the crypt shown to the right; these line profiles in actuality cover the entirety of the image. Images were then contrast-enhanced for increased efficacy in image segmentation. The pixel intensities are normalized to a range of 0 to 1, and contrast limit pairs have an upper and lower contrast limit, for example [0.2 0.9]. These contrast limit pairs refer to how 1% of image pixels fall below the lower contrast limit (0.2) and are mapped to a normalized signal value of 0, and 1% fall above the upper contrast limit (0.9) and are mapped
to a normalized signal value of 1. Contrast-enhanced images were then binarized with a
variable threshold automatically determined by Otsu’s method [31], designed to minimize
variance of values in the binary image. Figure 1(b) shows the effect of different contrast limit
pairs on binary images created for subsequent image segmentation. The optimal contrast limit
pairs will vary depending on the initial image intensity and contrast, therefore need to be
carefully selected for each topical contrast agent. In order to determine the optimal contrast
limit pairs for microendoscopy images of fluorescein-stained epithelium, 100 images of
porcine tissue were subject to a range of contrast limit pairs, to determine which pairs
produced the highest automated crypt detection sensitivity values [29]. Automated crypt
detection sensitivity was determined by comparing the sensitivity (true positives divided by
the sum of true positives and false negatives) of the algorithm to manual crypt identification
by a trained observer. Figure 1(c) shows the contrast limit pairs used to optimize the crypt
detection sensitivity; contrast limits were increased by 10% and ranged from [0.1 0.2] to [0.8
0.9].

Fig. 1. (a) Example of grid pattern on close-up of individual crypt (line profiles extracted every
40 pixels), a single pixel intensity line profile plotted as an example, and peaks and trough (red
arrows) labelled. (b) Example of effect of changing the contrast limit pairs on conversion into a
binary image. (c) Table of contrast limit combinations tested for automated crypt detection
sensitivity. Contrast enhancement scales image intensity values (0 to 255) between 0 and 1.
Both upper and lower contrast limits were iteratively increased by 10% to cover a range from
[0.1 0.2] to [0.8 0.9]. Gray region depicts impractical contrast limit pairs. Scale bars are
100\( \mu m \).
3. Results

3.1 Qualitative image comparison of topical contrast agents

Both topical contrast agents provide similar qualitative crypt structure information in microendoscopic images of superficial colon epithelium. Figure 2 shows a comparison of Figs. 2(a) and 2(b) fluorescein-stained, and Figs. 2(c) and 2(d) proflavine-stained, as well as Figs. 2(a) and 2(c) porcine, and Figs. 2(b) and 2(d) caprine epithelial tissue. Pathology, the gold standard, would use H&E staining to investigate the crypt shape, homogeneity, and nuclei shape and distribution [26]. The selected fiber bundle was designed in favor of a larger field-of-view, which inherently limits the resolution of individual nuclei. Using our system, neither proflavine nor fluorescein staining permit a careful study of nuclei shape and distribution, but they do highlight the shape and homogeneity of crypts. Both contrast agents delineate the inner crypts as dark, mostly homogenous, circular regions, while also providing contrast to the interstitial regions. While proflavine preferentially stains cell nuclei, leading to ring-like image features around the dark crypts (arrows in Figs. 2(c) and 2(d)), fluorescein stains the interstitial spaces more uniformly, while still providing contrast with the crypts (black arrows in Figs. 2(a) and 2(b)). In our experiments, fluorescein had an increased likelihood to pool at the center of the shallower caprine crypts [Fig. 2(e)], and appears as bright dot-like structures in the center of the crypts (yellow arrow in b).

![Fig. 2. Qualitative comparison between microendoscopic images of bulk colorectal porcine and caprine tissue stained with proflavine and fluorescein.](image)

When imaged under a 40x 0.8NA objective for a 261μm by 261μm field of view, using multiphoton microscopy, the subtle differences in staining are readily apparent. Fluorescein-stained bulk tissue [Fig. 3(a)] shows even staining of general intracellular material, as well as...
greater pooling in the center of the crypts. As expected, since proflavine intercalates DNA [19], proflavine-stained bulk tissue [Fig. 3(b)] preferentially stains individual nuclei. An example is shown in the inset of Fig. 3(b), of an individual nucleus, where the red arrows point to the nuclear membrane. Figure 4 shows the staining of both contrast agents at up to 100μm below the epithelial surface, which is more than sufficient for an end-on fiber bundle system that gathers data from the epithelial surface. This panel of depth sections additionally serves to exemplify how topical application of these contrast agents might also be applicable for depth-sectioning imaging modalities such as confocal microendoscopy.

Fig. 3. Qualitative comparison between multiphoton images of bulk colorectal caprine tissue stained with (a) fluorescein and (b) proflavine. (a) Tissue stained with fluorescein (0.04% (w/v) in distilled water). Yellow arrow points to fluorescein pooling in the center of the crypt. (b) Tissue stained with proflavine (0.01% (w/v in PBS)); white box inset shows a single stained cell, with yellow arrow pointing to the nuclear membrane. Both images were taken at 100 cumulative frames, 15fps, 20mW at sample, and have been equally enhanced for publication. Red scale bar is 100μm.

Fig. 4. Qualitative comparison of multiphoton images of bulk colorectal animal tissue stained with fluorescein and proflavine at 20μm, 60μm, and 100μm below the epithelial surface. Caprine tissue stained with fluorescein (0.04% (w/v) in distilled water). Porcine tissue stained with proflavine (0.05% (w/v) in 1x PBS). All images were taken at 100 cumulative frames, 15fps, 20mW at sample, and have been enhanced for publication. Scale bar is 100μm.
3.2 Image intensity as a measure of topical contrast agent

Qualitative comparison of matching weight-per-volume of proflavine and fluorescein showed that while increasing proflavine concentration increased signal intensity, the signal of the interstitial tissue was not as large in comparison to fluorescein. Quantitative results [Fig. 5] supported this: average image intensity of fluorescein was significantly brighter than proflavine at each individual concentration. There was little qualitative difference between fluorescein at 0.01% (w/v) and proflavine at 0.1% (w/v), which is supported by a quantitative lack of significant difference between the average image intensity of these solutions.

![Fig. 5. Comparison of different concentrations of topical contrast agents. Three different concentrations of each contrast agent, fluorescein and proflavine, were prepared at 0.01% (w/v), 0.05% (w/v), and 0.1% (w/v) in 1x PBS. Average image intensities (N = 10 images) of porcine colorectal tissue were plotted; error bars denote standard error. Fluorescein was significantly brighter than proflavine at each individual concentration, but fluorescein at 0.01% (w/v) was not significantly different from proflavine at 0.1% (w/v); statistical values were obtained from post-hoc testing of a one-way ANOVA using Tukey’s honestly significant difference (HSD), with p-value <0.05 as the criterion for significant difference. Microendoscopy images on the right are shown for qualitative comparison, and have not been contrast-enhanced; scale bar is 100μm.](image)

3.3 Optimization of contrast limit pairs for increased crypt detection sensitivity

As previously stated, a range of contrast limit pairs [Fig. 1(c)] were applied to 100 images of fluorescein-stained porcine tissue. The average sensitivity (defined as the number of true positives divided by the sum of true positives and false negatives, as compared to manually selected crypts) of each contrast limit is shown in Fig. 6 as heatmaps for 6(a) proflavine and 6(c) fluorescein stained tissue. The internally numbered boxes were labelled for ease of correlation of contrast pairs chosen to the Figs. 6(b) and 6(d) plots of average sensitivity at various combinations of these chosen and labeled pairs; for example, in Fig. 6(a), value 1 stands for contrast limit pair [0.5 0.9]. As described in our previous work [29], a combination of several contrast limit pairs dramatically improves the average sensitivity of our crypt detection algorithm. Based on the results of Fig. 6(b) we chose a combination of 5 contrast limit pairs for optimal crypt detection sensitivity of images of proflavine-stained tissue, as the sensitivity surpasses 80%. Based on the results of Fig. 6(d), we chose a combination of 3 contrast limit pairs for optimal crypt detection sensitivity of images of fluorescein-stained tissue, as the sensitivity surpasses 90% while reducing risk of overtraining.
Fig. 6. Optimization of crypt detection sensitivity by combination of contrast limit pairs for porcine tissue stained with (a) and (b) proflavine (0.01% (w/v)) and (c) and (d) fluorescein (0.04% (w/v) in PBS). (a) and (c) Heatmaps of the average percent sensitivity for each contrast limit pair; colorbars are average percent sensitivity. As with Fig. 2, the x-axes display the upper contrast limits, and the y-axes display the lower contrast limits. (b) and (d) Average test sensitivity, after 10 iterations of fivefold cross-validation, for each contrast limit pair or combination of contrast limit pairs. Maximum standard deviation of averages among all points was <0.61%. Heatmaps (a) and (c) are internally numbered with the selection of the highest sensitivity values per row, for clear correlation of numbered contrast limit pairs to the x-axis combinations in plots (b) and (d).

3.4 Quantitative image comparison of topical contrast agents

Our crypt quantification algorithm (QFEA) provided similar results for average crypt detection sensitivity in images of proflavine and fluorescein stained porcine tissue. As shown in Fig. 7, the QFEA was able to identify the majority of crypts for both topical contrast agents. One hundred images of proflavine-stained porcine epithelium and 100 images of fluorescein-stained porcine epithelium were quantified (QFEA) using contrast limit pairs selected using Fig. 6. As described in Section 2.7, a global QR value (peak-to-trough ratio) was calculated for each image as a metric of signal to background contrast, and the average QR of all images in a data set were tabulated against the average crypt detection sensitivity of
QFEA. In Fig. 8(a), the sensitivities and positive predictive values (PPV) of images with a QR value equal to, or higher than, the specified QR cutoff value (x-axis) were averaged for an aggregate average sensitivity at that QR cutoff. The sensitivity was previously defined as true positives divided by the sum of true positives and false negatives. PPV was defined as true positives divided by the sum of true positives and false positives. The peak aggregate sensitivity for proflavine occurred at a QR cutoff value of 1.08 [Fig. 8(a)]. QR cutoff values higher than 1.1 averaged less than five images for average sensitivity and PPV calculation of proflavine-stained images, and are therefore not reliable indicators due to undersampling. Average sensitivity of fluorescein-stained images continued to increase slightly for QR values higher than 1.1, while PPV had a slight decline after that point (not shown). It is of interest to note that images of proflavine were overall less sensitive to automated crypt detection, but had a markedly greater PPV. Fluorescein had a PPV that barely surpassed 70%, while proflavine had a PPV close to 90%. In contrast, fluorescein demonstrated average sensitivities consistently above 90%, while proflavine sensitivity peaked at almost 85% at a QR cutoff value of 1.06.

Fig. 7. Comparison of automated crypt detection between microendoscopy images of bulk colorectal caprine tissue stained with (a) fluorescein and (b) proflavine. Yellow asterisks mark the centroids determined with the QFEA. (a) Tissue stained with fluorescein (0.04% (w/v) in distilled water). (b) Tissue stained with proflavine (0.01% (w/v in PBS)). Both images have been equally enhanced for publishing. Scale bar is 200µm.

Fig. 8. (a) Average sensitivity and positive predictive values (PPV) of the QFEA crypt detection versus the QR cutoff value; aggregate performance plotted against QR was calculated using images with QR values equal to or higher to the QR cutoff value labeled in the x-axis. PS: Proflavine Sensitivity; PP: Proflavine PPV; FS: Fluorescein Sensitivity; FP: Fluorescein PPV. (b) Percentage of images with QR values equal to or higher than the labeled QR cutoff value; one hundred images per group. F: Fluorescein; P: Proflavine.
3.5 Quantification of crypt area and circularity in preliminary clinical trial

Automated quantification of crypt area and circularity, using our quantification algorithm (QFEA), was tabulated for regions with grossly normal, and adenocarcinoma diagnoses. 66 images, corresponding to approximately 25mm$^2$ of epithelial surface, were acquired from grossly normal regions (no dysplasia, no benign disease), and 76 images (~29mm$^2$) from adenocarcinoma regions. Figure 9 shows that there was significant difference between normal (N.) and adenocarcinoma (A.) regions, with a p-value of 0.035 for area, but circularity was not significantly different with a p-value of 0.13, using a Student T-test.

![Fig. 9. Quantitative comparison of crypt area and circularity. (a) Example of microendoscopic images of normal and adenocarcinoma regions. Scale bar is 100μm. (b) Average crypt area for normal and adenocarcinoma regions (N = 416 crypts, N = 603 crypts, respectively). P-value = 0.035. (c) Average crypt circularity for normal and adenocarcinoma regions (N = 416 crypts, N = 603 crypts, respectively). P-value = 0.13](image)

4. Discussion

Imaging of gastrointestinal epithelium via end-on fiber microendoscopy has been explored using DNA intercalating dyes, which so far have been used as investigational drugs in the United States [4,14,29]. A fluorescence alternative would be dyes already in clinical use, such as sodium fluorescein solution [32]. Our qualitative comparison of microendoscopy images of bulk colorectal tissue stained with fluorescein versus proflavine demonstrated that while the subcellular staining patterns differ [Fig. 3], fluorescein is able to provide similar information of crypt morphology to the previously established proflavine [Fig. 2]. An additional benefit of fluorescein as a contrast agent, in comparison to proflavine, includes reduced exposure time for images. Images of fluorescein-stained tissue were initially acquired at 100ms, for identical integration time of proflavine-stained tissue, but the images were over-saturated and we lowered the integration time until the images were of equal qualitative quality to proflavine. This resulted in fluorescein-acquisition at 50ms and proflavine-acquisition at 100ms. Reducing the required integration time has the benefit of reducing the number of motion-
blurred images. Reduced exposure time and reduced motion-blur is an important advantage for manual or in vivo acquisition, allowing for the flexibility of manually holding the probe yet not requiring the clinician to have steady hands. While clamping the probe during image acquisition would have eliminated motion from unstable handheld placement, we chose to retain the potential of motion-blurring in order to mimic in vivo imaging, where patient breathing or movement could contribute to motion-blur.

Quantitatively, the results of automated crypt detection demonstrated a sharp increase in crypt detection sensitivity using less combinations of contrast-limit pairs; while proflavine images required a combination of five contrast limit pairs to achieve greater than 80% average sensitivity, fluorescein images required a combination of only three contrast limit pairs to achieve an average sensitivity greater than 90% [Fig. 6]. A reduced number of contrast limit pairs is computationally time efficient, as well as reducing the risk of overtraining the algorithm. In addition to contrast limit pair combinations, the average sensitivity of the crypt detection algorithm is greater for the fluorescein data set. Even excluding images with low signal-to-background contrast (at a QR cutoff value of 1.06, Fig. 8(a)), the average crypt detection sensitivity of the proflavine data set never reached 90%, whereas the fluorescein data set average sensitivity was consistently greater than 90%. Despite this, the fluorescein data set has a consistently lower positive predictive value (PPV) [Fig. 8(a)]. Heatmaps of the PPV (data not shown) showed similar results to the sensitivity heatmaps, sharing the top two contrast limit pairs as resulting in the highest sensitivity and PPV values. This suggests that quantitative analysis of fluorescein images is more sensitive in the detection of crypts, while not as adept as a diagnostic tool due to a higher number of false positives, thought this requires additional investigation into alternative reasons for this imbalance.

Quantitative crypt detection results suggesting a lower PPV are not necessarily a critical limitation for clinical translation of a microendoscopic system. Given the length and size of the colorectal tract, there is minor risk in extracting multiple biopsies and therefore the surgical recommendations is to biopsy two to four regions each 10cm, with additional sampling of suspicious regions [33]. A highly sensitive, if not specific, tool would be useful in improving biopsy targeting of abnormal regions. While microendoscopic systems have achieved high sensitivity and specificity rates [14], they have not yet proven capable of replacing the gold standard of pathological staging of biopsies, therefore microendoscopy remains most useful in complementing existing endoscopy procedures. Our preliminary clinical results show that the automated algorithm measures a significant difference between crypt areas of normal and dysplastic regions, and additional work could increase the accuracy and breadth of quantifiable image features. One drawback of automated quantification, though, is that there is usually an increased regulatory burden when a system uses computer-based diagnosis, which is not an attractive prospect for vendors.

Fluorescein is also more relevant as a clinical contrast agent, compared to DNA-intercalating dyes, since it is already clinically used for intravenous injection and has demonstrated to pose limited risk to patient health. A drawback that should be considered is that intravenous fluorescein has a risk of anaphylaxis, with death from complications after intravenous injection during angiography procedures estimated at 1 in 220,000 patients [34]. Analysis of intrathecal administration of fluorescein in Europe and the United States showed that when a fluorescein dose of 1mL per 10kg of body weight of 5% (w/v) was used (total 50mg), side effects were minor and transient, with additional complications reported for higher doses (total 100-700mg) [35]. The side-effects of intravenous injection for confocal endomicroscopy have been similarly reported as minor for doses of up to 5mL of 10% (w/v) fluorescein, and while participants in their study experienced no severe reactions, the authors caution that anaphylaxis is rare but should be “anticipated and prepared for as for any drug [23]”. It is unclear if a topical application of the stain could possibly have reactions of equal or lesser severity. Alternatively, topical application of fluorescein required only the lower
recorded concentrations of fluorescein, and could potentially pose little risk to the patient in small topical doses.

Despite some limitations, we have described in this manuscript how topical application of fluorescein holds promise as a potential contrast agent alternative to proflavine for glandular tissue, specifically colon, since the crypt structures visible using proflavine are also visible when imaging with fluorescein. Using a 1mm fiber bundle, images acquired with both proflavine and fluorescein contrast offer similar image features for quantification, such as crypt area and circularity. There was over 90% crypt detection sensitivity of porcine tissue, as well as statistical significance in our preliminary clinical study using topical application of fluorescein solution, between grossly normal and grossly dysplastic regions of colorectal tissue.

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