Detection of colonic inflammation with Fourier transform infrared spectroscopy using a flexible silver halide fiber

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Abstract: Persistent colonic inflammation increases risk for cancer, but mucosal appearance on conventional endoscopy correlates poorly with histology. Here we demonstrate the use of a flexible silver halide fiber to collect mid-infrared absorption spectra and an interval model to distinguish colitis from normal mucosa in dextran sulfate sodium treated mice. The spectral regime between 950 and 1800 cm^{-1} was collected from excised colonic specimens and compared with histology. Our model identified 3 sub-ranges that optimize the classification results, and the performance for detecting inflammation resulted in a sensitivity, specificity, accuracy, and positive predictive value of 92%, 88%, 90%, and 88%, respectively.

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OCIS codes: (170.6510) Medical optics and biotechnology, spectroscopy tissue diagnostics; (300.6300) Spectroscopy, Fourier transforms; (110.2350) Fiber optics imaging; (070.4790) Spectrum analysis; (070.5010) Pattern recognition;

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1. Introduction

Ulcerative colitis (UC) and Crohn's disease are inflammatory processes in the colon that significantly increase the risk for development of colorectal cancer [1]. Unlike sporadic carcinoma, colitis-associated cancer develops from non-polypoid mucosa and follows an inflammation-dysplasia-carcinoma sequence [2]. In addition to the duration and anatomic extent of the disease, the severity of microscopic inflammation over time is also a risk factor in patients with long-standing inflammatory bowel disease (IBD) [3]. Thus, there is a clinical need to monitor the degree and severity of colonic inflammation [4]. Clinical indices, such as the Powell-Tuck Activity Score, do not correlate well with disease activity [5]. Moreover, endoscopy with biopsy is not effective for localizing microscopic inflammation in normal appearing mucosa on endoscopy. Furthermore, an assessment of inflammation throughout the various anatomic segments of the colon is important for evaluating the effectiveness of new drugs. Thus, a novel diagnostic technique that can perform a rapid assessment of inflammation in the colon would have great clinical potential.

The absorption of infrared light by the inter-atomic bonds in tissue biomolecules can be evaluated using Fourier transform infrared (FTIR) spectroscopy [6–10]. The characteristic spectra of these absorption peaks provide a molecular “fingerprint” that can be used to perform histological classification of tissue [11]. Recently, flexible endoscope-compatible optical fibers have been developed with silver halide materials that can be used to collect infrared spectra remotely [12–14]. Silver halides have features that provide significant advantages for *in vivo* use, including broad spectral transmission, good mechanical flexibility, low optical attenuation, long term stability, and no tissue toxicity.

Mouse models are important for the study of colitis, and have made significant contributions to our understanding of inflammation pathways, cancer transformation, and chemoprevention strategies [15–17]. Dextran sulfate sodium (DSS) mouse models have been shown to replicate important immunological and histopathological aspects of IBD that occur in human disease. The role of inflammation and the effect of disease activity on the development of cancer in this model have been accurately correlated with disease severity.

Here, we aim to demonstrate the use of this fiber material to collect infrared spectra for detecting the presence of mucosal inflammation in the DSS animal model of colitis. We also aim to show that FTIR is sensitive to changes in tissue biochemistry that occur in inflammation prior to the appearance of histological findings. Moreover, we aim to show that the diagnostic performance can be preserved with use of only 3 wavenumbers from the fingerprint region, allowing for a future laser-based system to be developed as a part of a clinical endoscope-compatible instrument.

2. Methods

2.1 DSS colitis model

Male C57/BL6 mice with age ranging between 8 to 10 weeks were obtained from Charles River. After 3 days for acclimatization, the animals were organized into 2 groups: 1) colitis (DSS) and 2) normal (control). A total of 16 animals (8 in each group) were used. Inflammation of the colon was induced in the colitis group by adding 2% dextran sulfate (MP Biomedicals, Solon, OH) to the drinking water for 7 days followed by 1 day of regular drinking water. The strength of DSS and the duration of therapy was based on our previous experience in order to induce optimum colonic inflammatory changes. The control animals were given only the regular drinking water. The animals were weighed and the feces were checked for occult blood daily. On day 8, the mice were euthanized by CO₂ inhalation. Afterwards, the full length of the colon was dissected intact, divided longitudinally to expose the mucosal surface, and rinsed with phosphate buffered saline (PBS) to remove debris. The right (R), transverse (T), and left (L) anatomic segments of the colon were identified, and the specimens were kept on ice during transport to the spectrometer. After the spectra were collected, the specimens were immersed in formalin for 24 hours, embedded with paraffin, sectioned and stained with hematoxylin and eosin (H&E). All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

2.2 Collection of FTIR spectra

The colonic specimens were placed onto a slide, and a grid overlay was used to achieve accurate localization of the sites measured. Spectra were obtained after reducing the effect of water absorption in the collected spectra by gently blowing cool air onto the mucosal surface for several seconds to remove ambient moisture. A spectrometer (Nexus 470, Thermo Electron, Madison, WI) with a mercury cadmium telluride (MCT) detector cooled with liquid nitrogen was used. The detection scheme is based on a Michelson interferometer, shown in Fig. 1, where a moving mirror varies the length of one optical path relative to the other, and creates an interferogram that is mathematically converted to an absorbance spectrum by a Fourier transform. The spectrometer was continuously purged with air to remove water vapor and CO₂. A 900 μ m diameter silver halide fiber is used in the attenuated total reflectance

configuration resulting in a total length of 1 meter (Multi-Loop-MIR™, Harrick Scientific, Pleasantville, NY). In the distal tip of the instrument, the fiber is arranged in a loop (diameter of 6.4 mm) that is placed into gentle contact with mucosal surface of the specimens for collection of mid-infrared absorption spectra. A gain of 4 was used to record the spectra with a resolution of 4 cm^{-1} using 36 co-added scans for both the background and tissue. Wavenumbers less than 900 cm^{-1} and greater than 1800 cm^{-1} were not collected because of the relatively high absorbance of silver halide outside of this range. Spectra were collected from the right (R), transverse (T), and left (L) anatomic segments of the colon from each mouse.

Fig. 1. **FTIR instrument.** Flexible silver halide fiber inserted into signal arm of Michelson interferometer is used to collect infrared (IR) spectra. Details provided in text.

2.3 Processing and classification of FTIR spectra

The FTIR spectra were classified using partial least squares (PLS) discriminant analysis [18,19]. The classification models were implemented using the SIMPLS algorithm in PLS_Toolbox, (Eigenvector Research, Inc, Wenatchee, WA) [20,21]. An interval model was developed that uses continuous regions of each spectra. This model determines the best performance for classifying the DSS and normal colonic segments using the complete spectral data set. Also, a discrete model was developed to use only 3 wavenumbers from each spectra to design a future clinical instrument with a laser source.

2.4 Interval model

In this method, the spectra were first pre-processed to remove sources of variance that are not needed for classification. First, interval PLS discriminant analysis was employed by dividing the spectra into six sub-ranges and identifying the presence of a subset of wavenumbers that provides a superior predictive value in comparison to use of the full spectral regime. All combinations of the sub-ranges were evaluated, ranging from a minimum of 78 spectral values to a maximum of 200. An exhaustive search of all combinations of spectral intervals was performed. An along-spectrum derivation was performed by taking the second derivative of each spectrum using the Savitsky-Golay algorithm [22]. A second-order polynomial was fit to a window around each value of the spectrum, and the value was then replaced with the second-order coefficient of this polynomial. Multiple window widths were tested because longer windows have a greater smoothing effect on the estimate of the second-derivative. Baseline subtraction was performed by first calculating a low-order polynomial that fit to the end regions of each spectrum and then subtracting this polynomial from the original spectrum.

Normalization was accomplished by ratioing each value of the spectrum with the area-under-curve (AUC) of the spectrum to achieve a unit area. Unit-variance scaling was performed by taking the value at each individual wavenumber in the spectrum and subtracting out the mean value from all of the spectra at that same wavenumber. Scaling to unit variance is then performed by dividing the result by the standard deviation. A variable-importance-in-projection (VIP) analysis was then performed from the PLS model weighting factors to evaluate the relative contribution of each wavenumber to the performance of the classification model, where the average wavenumber has a VIP value of 1 [23].

2.5 Discrete model

In this method, interval PLS discriminant analysis was employed as described above but using only combinations of 3 individual wavenumbers from each spectrum. Because of the large number of possibilities, a subset of 25 candidate wavenumbers was first identified from a VIP analysis. First, a PLS discrimination model using the spectral absorbance from all of the wavenumbers was calibrated. Then, the 25 highest local maxima based on VIP score were selected. Pre-processing was performed as described above, and the PLS model with the best performance is reported below.

2.6 Evaluation of histology

Histology was evaluated to determine the severity of mucosal damage. A score for each specimen was provided using a standard colitis index (CI) [24]. The criteria are defined by 4 categories based on the extent of crypt damage, inflammation, sub-mucosal edema, and hemorrhage, and scored according to severity over a range from 0 to 3. The score for each category was multiplied by an extent factor, defined as 1 for <10-25%, 2 for 25-50%, and 3 for >50%, corresponding to the proportion of surface area of diseased colon. In addition, 4 points were added for transmural involvement.

3. Results

3.1 DSS colitis model

The DSS mice gained weight in the normal fashion in comparison to the control animals for the first 5 days. Over the next 3 days, the DSS mice began to lose weight down to ~90% of their initial values. In addition, physical signs of colitis, such as frank and occult blood, appeared within 5 days of DSS administration. On the other hand, the control group gained weight as expected, and had normal stools with negative occult blood tests.

3.2 Processing and classification of FTIR spectra

Representative FTIR spectra collected with the silver halide fiber from the DSS and control specimens of colon over the full spectral regime (900 to 1800 cm^{-1}) from the right (R), transverse (T), and left (L) anatomic segments are shown in Fig. 2, and demonstrate very good SNR. We identified the following 6 sub-ranges that optimized the interval model: 1) 1000-1188 cm^{-1} ; 2) 1188-1325 cm^{-1} ; 3) 1325-1485 cm^{-1} ; 4) 1485-1585 cm^{-1} ; 5) 1585-1696 cm^{-1} ; and 6) 1696-1774 cm^{-1} . These sub-ranges are denoted by the vertical dotted lines in Fig. 2. The spectra reveal numerous absorbance peaks over this regime. Prominent peaks in sub-range 1 include 1037 cm^{-1} ($C-O$ stretch vibration of deoxyribose) and 1088 cm^{-1} (symmetric PO_2^- stretch) from nucleic acids. Notable bands in sub-range 2 include 1240 cm^{-1} (coupled $C-N$ stretch to $N-H$ bend of amide III), and significant peaks in sub-range 3 include 1398 and 1454 cm^{-1} ($C-H$ bend in aliphatic side groups of amino acids). In addition, large peaks are seen at 1545 cm^{-1} ($C-N$ stretch and $N-H$ bend of amide II) and 1640 cm^{-1} ($C=O$ stretch of amide I). A complete list of peak assignments are summarized in Table 1.

Fig. 2. Representative FTIR spectra for DSS (red) and normal (black) colonic mucosa over fingerprint region (900 to 1800 cm^{-1}) from the right (R), transverse (T), and left (L) anatomic segments demonstrate very good SNR and reveal numerous absorbance peaks in 6 separate sub-ranges.

3.3 Interval model

The best interval model for distinguishing between the DSS and control spectra used sub-ranges 1 to 3 only, as shown in Fig. 3. Sub-ranges 4 and 5 were found to be sensitive to the presence of water, and eliminated in this model. First, the mean unprocessed spectra for the DSS (red) and control (black) groups for sub-ranges 1 to 3 are shown in Fig. 3(A). Next, the mean spectra obtained after pre-processing with along-spectrum derivation (Savitsky-Golay algorithm) followed by normalization with the area under the curve are shown in Fig. 3(B). These spectra demonstrate how pre-processing enhances differences between the two groups. The length of smoothing window used was 11 points ($\sim 42 \text{ cm}^{-1}$) for sub-range 1, 15 points ($\sim 58 \text{ cm}^{-1}$) for sub-range 2, and 7 points ($\sim 28 \text{ cm}^{-1}$) for sub-range 3. The spectra are further processed by unit-variance scaling, as shown in Fig. 3(C), resulting in greater enhancement of the class differences in all 3 sub-ranges. The mean regression coefficients over the spectral regime, shown magnified by a factor of 10 (blue), determine the relative contribution of each DSS and control value to the overall prediction. The VIP scores shown in Fig. 3(D) reveal the relative contribution of each wavenumber to the performance of the classification model. The horizontal dashed line with a value of 1 denotes the average contribution. Prominent peaks are seen between 1060 and 1130 cm^{-1} in sub-range 1, where vibrational modes for nucleic acids and glycoproteins are located. Also, significant peaks can be seen in sub-range 3 between 1360 and 1500 cm^{-1} , where vibrational modes for amino acids and glycolipids occur.

Fig. 3. Pre-processing of spectra prior to classification. (A) The mean unprocessed spectra for the DSS (red) and control (black) mice from sub-ranges 1 to 3. (B) Along-spectrum derivation (Savitsky-Golay algorithm) followed by normalization enhances spectral differences between groups. (C) Unit-variance scaling further amplifies spectral differences. (D) VIP score reveals relative contribution of each wavenumber to the performance of the classification model.

The processed spectra from the interval model were then evaluated using the PLS algorithm. The classification values for the 3 colonic segments, right (R), transverse (T) and left (L), are shown in Fig. 4(A) for each animal. The DSS specimens are shown on the left (red) and the controls are presented on the right (black). A classification value of 1.5 (dashed horizontal line) was used as the diagnostic threshold. The ratio of DSS samples above this value (22/24) corresponds to a sensitivity of 92%, and that of control specimens below (21/24) correspond to a specificity of 88%. The overall accuracy and positive predictive value was found to be 90% and 88%, respectively.

3.4 Discrete model

Fig. 4. Performance for $n = 8$ DSS (red) and $n = 8$ control (black) mice in right (R), transverse (T), and left (L) colonic segments using the classification value of 1.5 (dashed horizontal line) as threshold. (A) Best interval model produced a sensitivity, specificity, accuracy, and positive predictive value of 92%, 88%, 90%, and 88%, respectively. (B) Best discrete model using 3 wavenumbers of 1072, 1088, and 1740 cm^{-1} produced results of 92%, 83%, 88%, and 85%, respectively. (C) Colitis index (CI) scores from histology are shown for comparison.

The best 3 wavenumber discrete model for distinguishing between the DSS and control spectra was found to have absorbances at 1072, 1088, and 1740 cm^{-1} . These 3 wavenumbers correspond to the $C-N$ stretch of glycoproteins, symmetric PO_2^- stretch of nucleic acids, and $C=O$ stretch of phospholipids. The processed spectra from this model were then evaluated using the PLS algorithm. The classification values for each of the 3 colonic segments are shown in Fig. 4(B) for each animal. A classification value of 1.5 (dashed horizontal line) was used as the diagnostic threshold. The ratio of DSS samples above this value (22/24) corresponds to a sensitivity of 92%, and that of control specimens below (20/24) correspond to a specificity of 83%. The overall accuracy and positive predictive value was found to be 88% and 85%, respectively.

3.5 Evaluation of histology

Furthermore, the CI score evaluated from histology for each colonic segment is shown in Fig. 4(C) for each animal. The DSS mice had a mean CI score of 5.1, 26.4 and 20.4 for the right, transverse, and left colonic segments, respectively. In comparison, the control mice had a significantly lower mean CI score of 2.1, 4.1 and 2.7, respectively. Interestingly, less inflammation was observed in the right colon of the DSS mice compared to the transverse and the left colonic segments. As expected, no particular segment of the control specimens was more likely to be misclassified. Representative histology from the transverse segment of the DSS and control mice are shown in Figs. 5(A) and 5(B), respectively, scale bar 50 μm . The DSS sections show features of mucosal damage, including distorted crypts, dot hemorrhages, and mucosal edema. The control sections show regular-spaced crypts, no hemorrhages, and absence of edema.

Fig. 5. Histology (H&E). Representative sections from (A) DSS colitis shows features of mucosal damage, including distorted crypt morphology, dot hemorrhages, and edema, and for (B) control (normal colonic mucosa) shows regular-spaced crypts, no hemorrhages, and absence of edema, scale bar 50 μm .

The ROC curves for the performance of the interval (solid) and discrete (dashed) models, shown in Fig. 6, reveal only a small reduction in the AUC from 0.92 to 0.88 with use of only 3 wavenumbers in the classification algorithm.

4. Discussion

Here, we demonstrate the use of a flexible silver halide fiber to collect infrared spectra remotely from freshly excised colonic mucosa from DSS mice to detect the presence of inflammation. To the best of our knowledge, this is the first demonstration of the use of an optical fiber to collect infrared absorption spectra to identify biochemical changes associated with inflammation. This technique is promising because endogenous tissue biomolecules that affect inflammatory pathways can be assessed with very high signal-to-noise in a simple instrument. Moreover, there is a significant clinical need for new technologies that can monitor the presence of inflammation in the digestive tract, as white light endoscopy alone is not sufficiently sensitive. This is particularly true for assessing the proximal (right sided) extent of disease in ulcerative colitis where microscopic inflammation can be found in macroscopically normal appearing mucosa [25].

Previously, infrared spectra have been collected using a benchtop (non-fiber) microscope and used to detect spectral differences among normal, colitis, and cancer using formalin fixed, paraffinized specimens [26]. We also found significant biochemical differences between normal and colitis in the spectral range between 900 and 1185 cm^{-1} , representing carbohydrates, phosphates, and nucleic acids with 2 of 3 wavenumbers from our discrete model (1072 and 1088 cm^{-1}) coming from this regime. However, the previous study on sporadic colonic dysplasia in the absence of inflammation did not find significant spectral

differences between 1350 and 1475 cm^{-1} , concluding that the levels of proteins and lipids are similar in normal and colitis [14]. In this study, the high VIP scores in this wavenumber range, along with the inclusion of the 1740 cm^{-1} in the discrete model, suggest that the levels of proteins and lipids do change with the onset of inflammation. In this *ex vivo* study, the tissue was blown with air to minimize interference from water. For future *in vivo* use, a catheter can be passed down the instrument channel of a medical endoscope to deliver air.

Fig. 6. ROC curves show comparison of performance between the interval (solid) and discrete (dashed) models for detection of colitis with optical fiber, resulting in an AUC of 0.92 and 0.88, respectively.

The course of IBD is affected by the severity and extent of inflammation in addition to genetic and environmental factors. We used 2% DSS to generate physical signs of colitis such as weight loss and bloody diarrhea that replicates clinical findings seen in patients with inflammatory bowel disease. The degree of inflammation seen using this concentration of DSS is similar to that encountered in clinical practice but is not readily detected using conventional endoscopic imaging methods especially when the disease is mild or patchy. Future protocols with a lower dose of DSS may help characterize the spectral readings in a lesser degree of inflammation. Despite widespread use of potent immunomodulators, the ability of drugs to alter the natural history of this disease and to prevent complications, such as stricture formation, remains largely ineffective. Moreover, colorectal cancer is a serious complication that accounts for ~10% to 15% of all IBD related deaths. As inflammation is the key component that drives this process, our ability to distinguish inflamed from normal mucosa remotely, as demonstrated by this study, offers hope for improved clinical interventions in the future. While the diameter of the current fiber used is too large to pass through the biopsy channel of a standard medical endoscope, the size is compatible with an integrated instrument. Moreover, the current length is adequate for use in a flexible sigmoidoscope, and longer lengths can be implemented with a laser-based system.

In our previous study [14], the performance of FTIR for the detection of colonic dysplasia in the absence of inflammation was assessed using histopathology as the gold standard. Histopathology is also the accepted metric for assessing the presence of inflammation and the extent of mucosal damage. However, the design of the current experiment allowed for a different evaluation of the sensitivity of FTIR for detecting inflammation. We observed 6 DSS mice with a CI less than the mean value of the control mice [Fig. 4(C)], indicating that inflammation was not yet apparent on histology. However, 5 out of the 6 mice were correctly

identified as DSS mice on FTIR. Thus, FTIR may be sensitive to biochemical changes that are apparent before micro-anatomical changes can be seen on histopathology.

Despite the histology showing morphological changes, including crypt damage, sub-mucosal edema and hemorrhage, over a depth of several hundred microns, as shown in Fig. 5, the evanescent wave produced at the distal tip of the silver halide fiber interrogates the tissue over a depth of less than 1 micron and is not likely to be sensitive to micro-anatomical alterations. By identifying subtle biochemical findings associated with inflammation, we may achieve a more complete understanding of the pathophysiology of IBD. This technique may offer insights into important clinical findings seen frequently on endoscopy that cannot be explained by conventional histology alone. For example, the nature of skip lesions in Crohn's disease, the abrupt transition from normal to inflamed tissue in UC, and the focal nature of strictures and cancers in both diseases may be better understood with additional biochemical data.

Table 1. Band assignments for inter-atomic bonds found in common tissue biomolecules for primary mid-infrared absorption peaks in molecular fingerprint regime are shown (ν_s – symmetric, ν_{as} – asymmetric stretch). References cited are within 5 cm^{-1} of those found in this study

Wavenumber (cm^{-1})	Vibrational Band	Biomolecules	Reference
970	$C-O$ stretch	DNA	[6]
1026	$C-O-H$ bend	carbohydrates	[6]
1037	$C-O$ stretch	DNA	[30]
1051	$C-O$ stretch	DNA	[6]
1072	$C-N$ stretch	glycoproteins	[31]
1088	$\nu_s PO_2^-$ stretch	nucleic acids	[30]
1154	$C-O$ stretch	carbohydrates	[6]
1225	$\nu_{as} PO_2^-$ stretch	nucleic acids, phospholipids	[30]
1240	$C-N$ stretch $N-H$ bend	proteins (amide III)	[6]
1301	$C-H$ stretch $N-H$ bend	proteins (amide III)	[6]
1350	PO_2^- stretch	lipids	[32]
1370	$C-H_3$ bend	proteins, glycolipids	[30]
1398	$C-H$ bend	amino acids	[6]
1454	$C-H$ bend	amino acids	[6]
1545	$C-N$ stretch $N-H$ bend	proteins (amide II)	[6]
1640	$C=O$ stretch $N-H$ bend	protein (amide I) glycoproteins	[6,31]
1740	$C=O$ stretch	phospholipids	[30]

In clinical practice, therapeutic efficacy is evaluated by improvements in subjective, self-reported symptoms. These criteria probably reflect a resolution of gross pathological lesions such as stricture or fistula rather than healing of subtle mucosal injury. As a result, non-invasive disease activity index scoring systems are not widely used in clinical practice and have not been formally validated [27]. Recently, the importance of mucosal healing for assessing drug efficacy has been emphasized. Achieving this aim remains a challenge given the patchy nature of inflammation on endoscopy, variability of findings on histology, and subjectivity among pathologists. The numerous absorption peaks seen here in the fingerprint region contain a wealth of information about tissue biochemistry that cannot only identify the presence of inflammation but may also be able to assess the severity. Effective strategies that can be translated to the clinic will require an integrated approach for data acquisition, classification, and validation [28,29]. Issues related to routine clinical use of silver halide

fibers, including cost, sterility, and stability, will also need to be worked out. Thus, continued development of fiber-based IR spectroscopy may contribute to the evaluation of new drugs for treatment of IBD in the future.

Acknowledgments

Research supported in part by grants from the National Institutes of Health, including P50 CA93990 and U54 CA136429 (TDW), and the Department of Defense USAMRMC W81XWH-07-C-0086 (RW).