Characterizing intestinal inflammation and fibrosis in Crohn’s disease by photoacoustic imaging: feasibility study

HAO LEI,1 LAURA A. JOHNSON,2 SHENGCHUN LIU,3,4 DAVID S. MOONS,5 TENG MA,6 QIFA ZHOU,6 MICHAEL D. RICE,2 JUN NI,1 XUEDING WANG,4,7 PETER D. R. HIGGINS,2 AND GUAN XU7*

1Department of Mechanical Engineering, University of Michigan, Ann Arbor, MI 48109, USA
2Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109, USA
3College of Physical Science and Technology, Heilongjiang University, Harbin, 150080, China
4Department of Biomedical Engineering, University of Michigan Medical School, Ann Arbor, MI 48109, USA
5Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA
6Department of Biomedical Engineering, NIH Ultrasonic Transducer Resource Center, University of Southern California, Los Angeles, CA 90089, USA
7Department of Radiology, University of Michigan Medical School, Ann Arbor, MI 48109, USA
*guanx@med.umich.edu

Abstract: The pathology of Crohn’s disease (CD) is characterized by obstructing intestinal strictures because of inflammation (with high levels of hemoglobin), fibrosis (high levels of collagen), or a combination of both. The accurate characterization of the strictures is critical for the management of CD. This study examines the feasibility of characterizing intestinal strictures by Photoacoustic imaging (PAI) without extrapolation from superficial biopsies. Ex vivo normal rat colon tissue, inflammatory and fibrotic intestinal strictures in rat trinitrobenzene sulfonic acid (TNBS) model were first differentiated by a PA-US parallel imaging system. Surgically removed human intestinal stricture specimens were afterwards imaged by a multiwavelength acoustic resolution PA microscope (ARPAM). The experiment results suggest that PAI is a potential tool for the diagnosis of the diseased conditions in intestinal strictures.

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1. Introduction

Crohn’s disease (CD) is an autoimmune disease of the intestinal tract affecting 700,000 people in the United States [1–3]. The pathology of CD is characterized by obstructing intestinal strictures because of inflammation (with high levels of hemoglobin), fibrosis (high levels of collagen), or a combination of both [4, 5]. Inflammatory strictures are usually medically treated, while fibrotic strictures are irreversible and may have to be removed surgically. An estimated 70% of Crohn’s patients require at least one surgery for their disease [3]. 13% have an unremitting disease and 10% have a prolonged remission [2]. The accurate characterization of the strictures, especially the presence or absence of fibrosis, is therefore critical for the management of CD [6, 7].

The fibrotic stricture can be characterized by its: 1) increased collagen content in the submucosa (below tissue surface and maybe up to several millimeters in deep), and 2) the loss of stratified tissue architecture [8–10]. Conventional imaging technologies including Ultrasound (US) imaging [10], Computed Tomography (CT) [4, 5, 11] and Magnetic Resonance Imaging (MRI) [1, 9] have been attempted to identify fibrotic intestinal strictures by resolving the stratified architecture in the strictures. However the diagnostic accuracy is limited as these modalities cannot assess intestinal strictures at the molecular level. The standard assessment of CD strictures is largely extrapolation from superficial biopsies, in which an endoscope integrating a light source and a camera is inserted into the digestive tract. By observing the video feedback from the camera, the clinicians first localize the strictures in the intestinal tract. Small sample pieces are removed from the inner layer of the strictures for histopathology. However, due to the limited number of sampling locations and depth of endoscopic biopsy procedure, comprehensive evaluation of an intestinal stricture is hard to achieve [10].

The distinctive optical absorption spectra of collagen and hemoglobin (shown in Fig. 1) [12, 13] prompt the use of optical imaging approaches, such as diffuse optical spectroscopy (DOS) [14, 15] and optical coherent tomography (OCT) [16, 17] to access fibrosis and inflammation conditions. However, DOS may only evaluate the molecular components of the bowel wall without architectural information. Backscattering based OCT, with excellent spatial resolution, can resolve detailed bowel wall structures at limited depth without interrogating the molecular components [16, 17]. Photoacoustic (PA) imaging (PAI) is an imaging technique combining high optical sensitivity for discriminating the molecular contents and good ultrasonic resolution to observe microscopic architecture in biological tissues [18, 19]. PAI has demonstrated the capability to assess hemoglobin and collagen contents in biological tissue [12, 20]. PAI could therefore be a potential tool for the characterization of the diseased conditions in intestinal strictures. In addition, PAI can reach up to 7 cm below tissue surface at a resolution of 1/200 of the desired imaging depth [18]. The stratified tissue architecture in the bowel wall is on the order of tens of microns and is millimeters beyond the stricture inner surface [21, 22], which is ideally covered by acoustic resolution PA microscopy (ARPAM) [18].

This study examined the feasibility of characterizing the intestinal stricture using ARPAM. Our PA-US parallel imaging system was initially used to demonstrate the capability of PAI in quantitatively differentiating ex vivo normal intestine colon tissues, fibrotic and inflammatory intestinal strictures from trinitrobenzene sulfonic acid (TNBS) model in rat [22–24]. Surgically removed human intestinal specimens were afterwards imaged in an ARPAM system. Confirmed by histology, ARPAM is capable of resolving the spatial distribution and relative concentrations of molecular components in intestinal strictures.
Fig. 1. Relative optical absorption spectra of major chemical components in biological tissue [12, 13]. The vertical dashed lines mark the relatively higher optical absorption of hemoglobin, lipid and collagen at 532, 1220 and around 1310 nm, respectively. a.u. = arbitrary units. Hb. = Hemoglobin.

2. Methods

2.1 Quantitatively differentiating the diseased conditions in intestinal strictures in rat TNBS model

Three types of rat colon tissues were examined by PAI ex vivo, including the normal colon, the acute (pure inflammatory) and chronic (mixed inflammatory and fibrotic) inflammatory strictures. The normal colon and acute inflammatory intestinal stricture were compared by PAI at 532 nm due to the increase of hemoglobin content in acute inflammatory intestinal strictures. The acute and chronic inflammatory strictures were compared at 1370 nm due to the increase of collagen content in chronic inflammatory intestinal strictures.

2.1.1 Animal preparation for comparison between normal and acute TNBS model

Six female Lewis rats (Harlan, Indianapolis, IN) were divided into two groups. One group received a single 10 mg intrarectal dose of TNBS using the method described in [23]. This procedure produces acute inflammation of the distal colon which peaks 2-3 days after administration. A second cohort of 3 animals (negative control) received an intrarectal dose of Phosphate-buffered saline. Both groups were euthanized 2 days post administration. Distal colons were collected for PAI, histology, and molecular analysis.

2.1.2 Animal preparation for comparison between acute and chronic TNBS model

20 female Lewis rats were divided into two groups of 10 animals each. One group (acute TNBS, pure inflammatory) received a single 10 mg intrarectal dose of TNBS as described above. The second group animals (chronic TNBS, mixed inflammation and fibrosis) received weekly escalating doses of TNBS from 10 mg to 60 mg over 6 weeks as previously described [22, 23]. Acute animals were euthanized 2 days post administration. Chronic animals were euthanized 2 days after the last TNBS treatment. One animal in the chronic group died prior to the 6 week cycle and was not imaged. Distal colons were collected for PAI, histology, and molecular analysis.
2.1.3 Experiment setup for ex vivo PAI

The ex vivo bowel wall samples were flattened and cut into circular shape with diameters of 12.5 mm and fit into a gelatin made sample holder submerged in water (Fig. 2). Small amount of water was added on top of the sample to allow PA signal coupling along the radial direction of the sample holder. A tunable Optical Parametric Oscillator (OPO) laser (Vibrant B, Opotek Inc., Carlsbad, CA, USA) pumped by the second harmonic output of an Nd:YAG pulsed laser (Brilliant B, Quantel, Bozeman, MT, USA) provided the illumination laser for PAI. The output beam of laser was expanded and collimated to a cross-sectional diameter of 12.5 mm to cover the opening of the sample holder. Such light delivery scheme minimizes the attenuation of the optical energy by water at 1370 nm. The light density on top surface of samples at both 532 nm and 1370 nm was adjusted to approximately 7 mJ/cm², which is under the safety limit established by American National Standards Institute (ANSI). The PA signals were collected and processed by our PA-US parallel imaging system based on Verasonics US platform (Vintage, Redmond, WA) with a commercial US transducer array (L7-4 Philips Healthcare, Andover, MA, USA) [25]. The signals were averaged for 30 times for noise reduction.

![Fig. 2. Experiment setup for rat colon tissue imaging. The sample tissue specimen was flattened and laid at the bottom of a sample holder submerged in water. Laser was delivered from the opening of the sample holder. PA waves were coupled into the wall of the sample tube and propagate into water. An US probe acquires the PA signals. A PA-US imaging system acquires both the PA and US images of the samples.](image)

2.2 PAI of human intestinal stricture specimens

Surgically removed, deidentified human intestinal strictures were procured through the standard procurement procedures in the Department of Surgery. The capability of a multispectral ARPAM in resolving the distributions and concentrations of the molecular components in the intestinal strictures were examined.

2.2.1 Experiment setup of ARPAM

Figure 3 shows the schematics of the ARPAM system. The OPO laser in section 2.1 was used as the illumination source at 532, 1220 and 1310 nm targeting hemoglobin, lipid (correlated to edema accompanying inflammation) and collagen contents. The output beam of laser was collimated and coupled into a bundle of 9 multimode optical fibers (0.39 NA, 400 μm core, Thorlabs). One spherically focused transducer (48 MHz central frequency, 30 MHz...
bandwidth, 3.1 mm focal length, 3.5 mm aperture) received the acoustic signals. The transducer and the output ends of the optical fibers were securely fixed by a cone-shaped holder, which allows the adjustment of the directions of fiber tips for optimal illumination. The optic fibers were evenly distributed in a circle to formulate a ring illumination pattern, reducing PA signal intensity at the tissue surfaces and guaranteeing homogenized light fluence in deep tissue for narrower signal dynamic range [26]. The optical density on the tissue surface was maintained below ANSI safety limit for all wavelengths at approximately 16 mJ/cm². The cone-shaped holder was precisely driven by a 3D linear translation stage for 2D/3D raster scan. The PA signals were amplified by 30dB and recorded by an 8-bit digitizer card (DP1400, Agilent Tech, USA).

The human intestinal strictures were cut into pieces with areas of around 15 mm by 15 mm and fixed in a petri dish using porcine gel with the inner surface facing up. Small amount of water was added on top of the samples for acoustic coupling. B-scans perpendicular to the tissue surface with step size of 50 μm were performed to form 2D PA images of the cross-sections of the samples at wavelengths of 532, 1220 and 1310 nm. For each sampling location, PA signals were averaged 10 times to improve the signal-to-noise ratio (SNR). With a repetition rate of 10 Hz for the illumination source, the total scanning time was 15 minutes. Lastly, the scanned portions of the specimens were cut off for histology with H&E and Massons’ Trichrome staining.

2.2.2 Non-uniform response along depth dimension PA measurement

In order to compensate the non-uniform fluence distribution of light and spatial sensitivity of the US transducer, we performed Monte Carlo simulations [27] for each wavelength to estimate optical energy distribution in the sample tissues. In the simulations, we assumed that the tissue sample had a homogenous background consisting of widely distributed components such as water, lipid and hemoglobin. The changes of the molecular components characteristic of the diseased conditions were treated as perturbations and solved later [28]. The background optical properties were calculated based on the absorption spectra in Fig. 1 with the estimated concentrations of water, lipid and hemoglobin of 70%, 25% and 0.05%, respectively, and the scattering properties calculated following the approach in [29] (values listed in Fig. 4 captions). Low hemoglobin concentration was used due to the loss of blood during surgical sample collection. Using the estimated beam profile at sample surface shown in Fig. 4(A),
fluence distributions for wavelength of 532 nm, 1220 nm and 1310 nm were simulated, as shown in Figs. 4(B) to 4(D), respectively. The light fluence variations along the transducer focusing axis were plotted in Fig. 4(E).

The acoustic intensity map of the focused transducer was simulated based on Huygens’ Principle [30, 31]. As shown in Fig. 4(F), one-way acoustic intensity map of the focused transducer was simulated with central frequency at 48 MHz and bandwidth of 30 MHz. The acoustic attenuation was estimated as the case of typical soft tissues with 0.5 dB/cm-MHz [32].

2.2.3 Relative concentration of molecular components

In the PA images compensated by the profiles in Fig. 4, the magnitudes of the pixels, i.e. the intensities of the PA signals, should be proportional to the total optical energy absorption [33] at the wavelengths where the signals were acquired. The corresponding total optical absorption rate can be presented by:

$$A_i = \sum_j c_j \cdot \mu_{ji}$$  \hspace{1cm} (1)

where $A_i$ is total optical absorption rate at wavelength $i$, which is proportional to PA intensity; $\mu_{ji}$ is the absorption coefficient of molecular component $j$ (Hb: hemoglobin; cg: collagen; lp: lipid) at wavelength $i$, which can be found in previous studies [12, 13]; and $c_j$ is the relative concentration of molecular components $j$. As the contributions of hemoglobin, lipid and collagen at each pixel to the image magnitude of each pixel, the relative concentrations was inversely solved following Eq. (2) [28, 33, 34]:

$$
\begin{bmatrix}
  c_{Hb} \\
  c_{cg} \\
  c_{lp}
\end{bmatrix} =
\begin{bmatrix}
  \mu_{Hb,532} & \mu_{cg,532} & \mu_{lp,532} \\
  \mu_{Hb,1220} & \mu_{cg,1220} & \mu_{lp,1220} \\
  \mu_{Hb,1310} & \mu_{cg,1310} & \mu_{lp,1310}
\end{bmatrix}^{-1}
\begin{bmatrix}
  A_{532} \\
  A_{1220} \\
  A_{1310}
\end{bmatrix}
$$  \hspace{1cm} (2)
The relative concentrations of each molecular component were encoded in pseudocolor and coregistered formulating molecular components. Spatial correlations between the PA images and the histology photographs were found by a board certified anatomic pathologist who has extensive experience with the pathology of Crohn’s disease in human subjects. The correlations were achieved by determining unique areas of architecture present in both the H&E and pseudo-color PA molecular component images. This allowed the identification of areas of fibrosis and inflammation in the H&E section that could then be correlated with the area. Once landmarks were identified, the relative areas of fibrosis in both areas was then able to be compared.

3. Results

3.1 Statistical analysis of the PA measurements on TNBS rat models

Figure 5(A) shows the representative PA images of colon tissues from normal and acute TNBS rats at 532 nm. The high pixel intensity in the image of the acute TNBS sample is due to the significantly increased hemoglobin content. An averaged pixel intensity increased by 2.3 times (p<0.0001) in pure inflamed tissue comparing to normal control tissue, as shown in Fig. 5(C). With the data sets shown in Fig. 5(C), the PA approach has shown a power of 0.93 to differentiate the normal and the acute inflammatory colons in two tailed t-test with a confidence of 95%.

Figure 6(A) shows the representative photograph of acute and chronic intestinal strictures in TNBS rats. Figure 6(B) shows the corresponding US and PA images at 1370 nm. Figure 6(C) shows that the incidence of fibrosis (relevant to more collagen content) in the chronic inflammatory samples leads to a 2.9 fold increase in average pixel intensity (p<0.0001) against acute inflammatory samples. A power close to 1 with 95% confidence can be
observed in the two tailed t-test to differentiate the two types of tissue samples with the PAI data.

3.2 PA molecular component images of human intestinal stricture specimens

Figure 7(A) and 7(C) show the pseudo-color PA molecular component images for two human intestinal stricture specimens, respectively. The curvatures of the samples were distorted by cutting for histology. Perfect alignment between the histology photos and the PA molecular component images therefore can hardly be achieved. However, the stratified architecture of the samples was resolved and spatial correlation can still be found (pointed by the arrows in Fig. 7).
Fig. 7. Representative PA molecular component images (A)(B) and histology images (B)(D) of human intestinal strictures. MC: mucosal layer; MS: muscle layer. The specimens were scanned at 532, 1220 and 1310 nm. The relative concentrations of hemoglobin, collagen and lipid were inversely solved by Eq. (1) and encoded in (A) and (C) in red, green and blue, respectively. The color scales were normalized to the maximum concentration of hemoglobin. Both samples have collagen components, which is confirmed in (B)(D) with Masson’s trichrome staining. The inflammations in the samples were confirmed by H&E staining, as shown in the insertions in (B) and (D) in red boxes. The insertions were taken at 20x magnification. Spatial correlations between the histology and the PA images are marked by green arrows (high collagen concentration), red arrows and dashed contours (neovascular tissues containing hemoglobin), and blue arrows (lipid deposition caused by edema). The porcine gel for fixing the samples with very low collagen concentration was also stained in blue yet with uniform texture and marked by black arrows.
4. Discussions

The colon samples derived from TNBS rat model represent extreme diseased conditions. The severe fibrosis lead to extensive collagen deposition over the entire tissue volume and the destruction of the stratified architecture [22]. Our PA-US parallel imaging system with limited spatial resolution was therefore used for assessing only the molecular components of the samples. More animals were included for the comparison between acute and chronic TNBS model in seek of statistical significant results. This is because the optical contrast of collagen over hemoglobin at 1300s nm is much lower than the contrast of hemoglobin over collagen at 532 nm. Wavelength in 1300s nm range was used to compare acute and chronic inflammatory samples as collagen has positive optical contrast over hemoglobin. In addition, since hemoglobin and collagen have almost parallel optical spectra in 1300s nm, optical wavelengths within this range produce similar results as the major molecular components in the samples were limited to hemoglobin and collagen. Water component change in the two types of samples was not considered as we did not find diseased conditions correlated to water content change [22, 23]. Water content within the sample holder is stringently controlled to be identical for all experiment cases and thereby does not contribute to the difference between the PA images of the samples. The PA signal differences shown in Fig. 5(C) and Fig. 6(C) have proved the capability of quantitatively differentiating the extreme diseased conditions by PAI. Colon samples with gradually changed disease conditions will be tested in our future study for further understanding the sensitivity and specificity of PAI on characterizing the intestinal strictures. Although the pulse-echo US did not show significant differences between the samples, US elasticity imaging also under investigation by the authors has demonstrated promising results in distinguishing inflammatory and fibrotic intestinal strictures [22, 23]. Comparison between PAI and elasticity US as well as the effectiveness of diagnosis using both modalities will be investigated in our future study.

In the experiments on human intestinal stricture specimens, PAI at the three selected wavelengths has demonstrated reliable capability in resolving the molecular components of interest. In addition to the strong hemoglobin absorption at 532 nm, the comparable absorption coefficients of oxy- and deoxygenated hemoglobin at 532 nm also minimizes the differences between \textit{ex vivo} and \textit{in vivo} measurements caused by varied oxygenation levels. The wavelength of 1310 nm is chosen here for its relatively low optical absorption by water and lipid so that better imaging depth can be achieved and coupling from lipid signals can be minimized. The PA measurements are compensated by the optical energy distribution at each wavelength with the approximation of homogeneous optical properties in the samples. Collagen content, although with lowest optical absorption coefficient among the three molecular components studied, has been detected at depth of 6.5 mm in Fig. 7(C). The assumption of homogeneous background limited the quantitative accuracy of Fig. 7(A) and 7(C). Iterative methods calibrating the optical fluence within the tissue samples will be attempted in the future following previous studies [35, 36]. Water content was considered as part of the homogeneous background in the human tissue samples as water is extensively diffused within tissue and does not demonstrate significant positive optical contrast over the molecular components studied. Including more optical wavelengths to resolve addition molecular component such as water could improve the accuracy of the ARPAM system, which will also be investigated in the future.

The limited bandwidth of the US transducer was incapable of covering the full spectrum of PA signal power and could lead to the difficulty in determining the absolute concentrations of the molecular components. We thereby used relative concentrations for all cases. The mucosal layer and the top of muscle layer of the primary diagnostic interests are adequately covered by the focal zone of the transducer. The PA signals from the deep tissues have been compensated by the spatial sensitivity of the US transducer.

Although a prominent spatial correlation between the PA molecular component images and the histology images is found, the areas of the collagen and lipid do not perfectly match.
This owes to the limited deliverable optical energy, sensitivity of the US transducer and signal-to-noise ratio of the ARPAM system, in which weak PA signals generated by molecular components at low concentrations are overwhelmed. The ultimate implementation of the ARPAM approach for assessing intestinal strictures requires further optimization and miniaturization of the prototype system to an endoscopic imaging probe. The small spherically focused transducer ensured desirable resolution for observing histological features and the miniaturization. Once that endoscopic probe is successfully developed, such endoscopic PAI diagnostic strategy could facilitate quick and infinite number of samplings for comprehensive and longitudinal assessment of the strictures, leading to personalized therapy as well as improved understanding and management of CD.

5. Conclusions

This study validates the feasibility of PAI in assessing the molecular components and microscopic architectures of intestinal strictures in Crohn’s disease using ex vivo tissues from animal models and human subjects. A prototype ARPAM system was developed and has demonstrated the capability of noninvasively producing molecular component images in human intestinal strictures through superficial measurements.

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