Infrared spectroscopic imaging detects chemical modifications in liver fibrosis due to diabetes and disease

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Abstract: The importance of stroma as a rich diagnostic region in tissue biopsies is growing as there is an increasing understanding that disease processes in multiple organs can affect the composition of adjacent connective tissue regions. This may be especially true in the liver, since this organ's central metabolic role exposes it to multiple disease processes. We use quantum cascade laser infrared spectroscopic imaging to study changes in the chemical status of hepatocytes and fibrotic regions of liver tissue that result from the progression of liver cirrhosis to hepatocellular carcinoma and the potentially confounding effects of diabetes mellitus.

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1. Introduction

The liver is an organ with varied functions that both make it essential to the body's metabolism and expose it to harm from chronic disorders. The hepatocytes, parenchymal cells that constitute most of this organ, can be damaged by lifestyle factors such as alcoholism; infections such as hepatitis A, B, or C; or other diseases such as non-alcoholic steatohepatitis [1]. In order to survive such damage, the liver demonstrates remarkable regenerative abilities; hepatocytes can be quickly and repeatedly replaced following injury. However, repeated cycles of damage lead to inflammation and fibrosis.

Liver fibrosis consists of scarring of the hepatic tissue, as the organ accumulates extracellular matrix (ECM) components in the stroma, chiefly collagen. A complex disease process, believed to involve the activation of hepatic stellate cells and interactions amongst other cell types, leads to the massive deposition of collagen and other ECM proteins in a manner that disrupts the functional structure of liver tissue [1, 2]. The end result of this process is cirrhosis, a common end-point for liver diseases of varying etiology. At this stage, the loss of liver function on its own poses a dire threat to health; however, patients who have reached this point from many different hepatic diseases are also at risk of developing dysplasia, and ultimately hepatocellular carcinoma (HCC) [2, 3].

The complexity of the disease processes of fibrosis, cirrhosis, and hepatocarcinogenesis, coupled with the variable etiology of liver disease necessarily complicates any chemical imaging approach to the study of hepatic tissues. Comorbid conditions, in particular diabetes mellitus, can have an effect on disease progression and prognosis [4, 5]; it is possible that liver biopsies from diabetic patients may present distinct chemical signatures. Understanding these differences, when analyzing both regions of fibrosis and dysplastic or malignant hepatocytes in the context of HCC, is essential when aiming to derive useful diagnostic or prognostic information from chemical imaging of liver biopsies.

Infrared (IR) spectroscopic imaging is an approach to image tissues in a stain-free manner to derive biochemical information. This technique is based on the principle that different regions of the mid-IR range are absorbed by different chemical bonds that form the base constituents of key cellular biomolecules such as proteins, lipids, DNA, collagen, and glycogen that can be modified during disease. Previous work in the liver has demonstrated that damage and disease in this organ leads to changes in the IR absorption spectra of the tissue [6, 7].

We have also recently shown that different structures in the kidney are chemically modified due to diabetes and that these changes can be detected before morphological changes are apparent [8]. A number of recent review papers discuss the applications of IR imaging for tissue imaging [9]. A new advancement in mid-IR imaging has come in the form of tunable mid-infrared quantum cascade lasers (QCL) that allow for high-intensity discrete-frequency measurements and live imaging by exploiting the full laser brightness at selected wavelengths [10–13] which is potentially advantageous in deployment of IR imaging in the pathology clinical setting (Fig. 1).
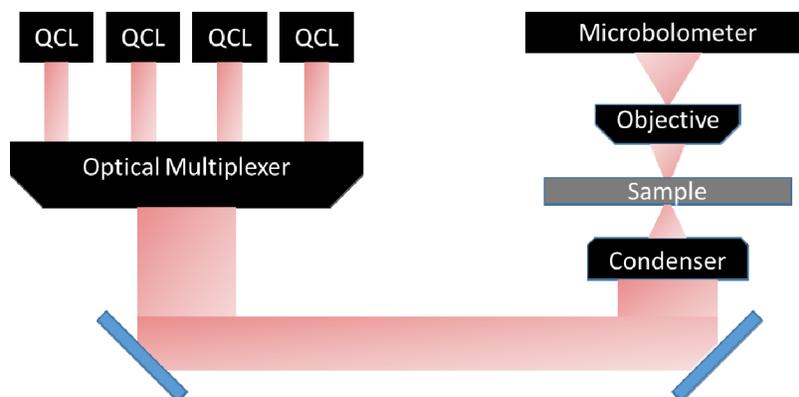
Fig. 1. QCL IR microscope schematic. The light source consists of four lasers that are combined by the optical multiplexer to allow for tuning and discrete-frequency imaging. The rest of the assembly functions in a manner similar to an optical microscope, and can be used for imaging of unstained tissues mounted on IR-compatible substrates.

2. Materials and methods

Samples were chosen from 2 formalin-fixed paraffin embedded tissue microarrays (TMAs) from the University of Illinois at Chicago tissue bank. The TMAs consisted of liver tissue biopsies from patients with various stages of liver disease, ranging from cirrhosis to hepatocellular carcinoma. Individual patient cores were selected on the basis of diabetic status as well as a visual analysis of hematoxylin-and-eosin (H&E) stained sections to confirm the presence of regions of both distinctive fibrosis and hepatocytes on the tissue samples. The H&E sections were also examined by a pathologist to determine the primary disease state displayed by the hepatocytes.

For infrared imaging, the TMAs were sectioned at 4 μm thickness with a microtome onto BaF_2 slides. The sections were deparaffinized by soaking in hexane for two days, replacing the solvent approximately every six hours [9]. From these, 10 diabetic and 10 non-diabetic patient cores were scanned with the Daylight Solutions Spero QCL imaging system on transmission mode (collecting wavenumbers 1800 to 900 cm^{-1}) using the 12.5X, 0.7 numerical aperture objective with a pixel size of 1.4 × 1.4 μm (field of view 650 × 650 μm). The scans were then exported to ENVI-IDL for processing, namely linear baseline correction between 1796 cm^{-1} and 904 cm^{-1} and normalization to the absorbance at 1652 cm^{-1}. Regions of interest were drawn by comparison to the H&E-stained sections for regions of fibrotic scarring and regions of hepatocytes, and pixel-level and averaged spectra were extracted for further analysis. Multivariate analysis techniques such as Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) were applied to the complete extracted spectra.

3. Results and discussion

IR imaging allows for the acquisition of images wherein each pixel comprises a mid-infrared spectrum. This means IR tissue images display contrast based on their inherent biochemistry; for example, in the liver, regions of hepatocytes and fibrosis can be highlighted by using single discrete frequencies for visualization (Fig. 2). With new QCL based approaches, it is possible to tune specifically to spectral frequencies of interest, permitting ready identification of tissue features of interest. This may be useful for pathologists when examining liver biopsies in order to quickly highlight different structural aspects of the tissue. It can also allow for rapid collection of a handful of wavenumbers of interest based on their biochemical significance, and visualizing their spatial patterns across a tissue sample.
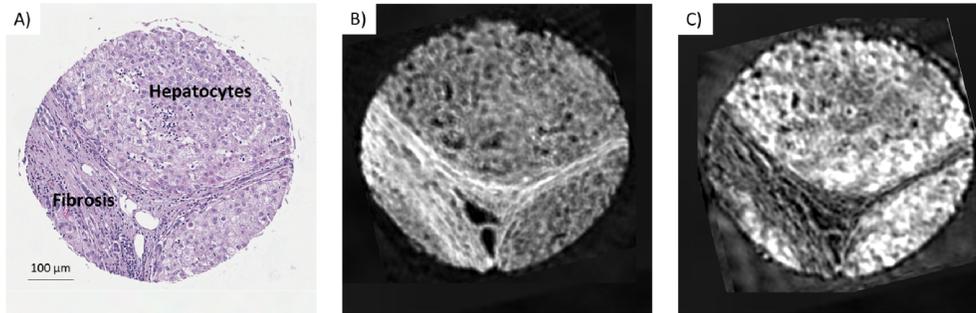
Fig. 2. A) H&E-stained section from a liver tissue biopsy, with regions of hepatocytes and areas of fibrosis indicated B) An unstained section from the same biopsy scanned in IR, presenting absorbance at 1656 cm^{-1} C) Same section as in (B), presenting absorbance at 1032 cm^{-1}.

IR spectroscopic imaging can easily discriminate between the two major components of liver biopsies, hepatocytes and fibrosis, using a simple supervised classifier such as Principal Component Analysis coupled with Linear Discriminant Analysis (PCA-LDA) (Fig. 3(B)). One important note is that the average spectra derived from each patient core from pixels associated with hepatocytes showed a large degree of spectral variance in the region from 1200 cm^{-1} to 1000 cm^{-1} (Fig. 3(A)). The high degree of intra-class variance demonstrated in the hepatocytes may be a source of concern as it can mask important spectral variance for disease typing in a similar fashion to what was found in early work using IR spectroscopy of exfoliative cervical cytology [14]. The source of this intra-class variance will most likely be due to two sources; accumulation of glycogen [15] in the liver as part of glucose homeostasis (which may be affected by diabetes) and contribution from clear cell inclusions as part of liver disease. The clear cell inclusions are often found in hepatocytes in liver disease and may consist of different components such as glucose, fats, and water. Future work will focus on determining whether these tissue entities, despite being potential confounding factors, offer strong prognostic information.
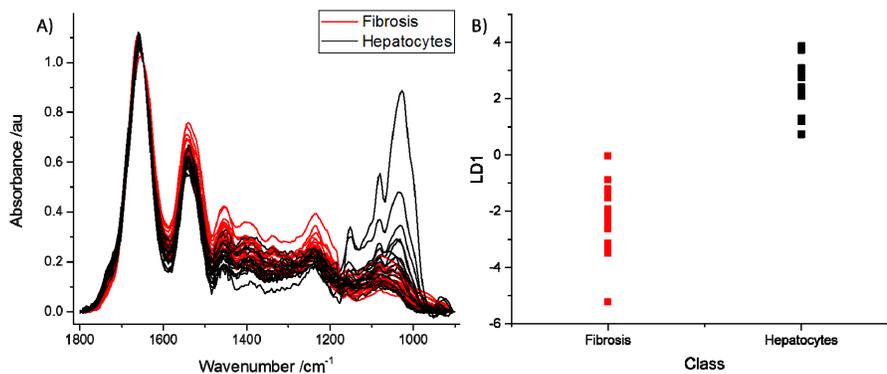

Fig. 3. A) Average spectra from regions of fibrosis and hepatocytes across both diabetic and non-diabetic patients, show that there significant variability for hepatocytes in the region from 1200 cm^{-1} to 1000 cm^{-1}.B) Using PCA-LDA, hepatocytes and fibrosis can be readily distinguished.

Another potential confounding factor is diabetes mellitus, a disease whose rate in western society is booming and carries with it a significant economic and social burden. Diabetes can cause a range of co-morbidities such as diabetic nephropathy, diabetic retinopathy, and diabetic neuropathy. The disease itself is caused by the loss of the body's ability to regulate glucose levels in the blood stream, specifically to remove glucose when the concentrations are elevated after consuming carbohydrates. This leads to excess glucose binding to proteins in

tissues, in particular endothelial cells, by a process known as glycation which can impair the function of cells and organs. Overall, diabetes is an important risk factor associated with worse outcomes in liver cirrhosis and hepatocellular carcinoma [4, 5, 16].

To explore the impact of diabetes on the liver, this study investigated whether patients with diabetes had different biochemical profiles in either their hepatocytes or in the regions of infiltrating fibrosis. IR images were acquired from 20 patients, 10 with diabetes and 10 without diabetes. IR spectra were extracted from the regions of hepatocytes (Fig. 4(A)) and the regions of fibrosis for each patient (Fig. 4(B)) and compared. PCA-LDA of hepatocyte derived spectra demonstrated modest separation between the two classes with 3 patients overlapping (Fig. 4(D)) whereas in the regions of fibrosis clear separation between the two classes could be achieved (Fig. 4(E)). This is somewhat anticipated as previous work in diabetic nephropathy demonstrated that the two spectral frequencies most altered due to diabetic damage were found at the spectral peaks 1030 cm^{-1} and 1080 cm^{-1}, which in the case of hepatocytes are swamped by intra-class variance. In fibrosis these confounding factors do not exist and as such there is an ability to better discriminate due to the availability of these frequencies for classification (Fig. 4(C)). When all four classes were classified using PCA-LDA separation between cell type and diabetic status could be achieved (Fig. 4(F)).
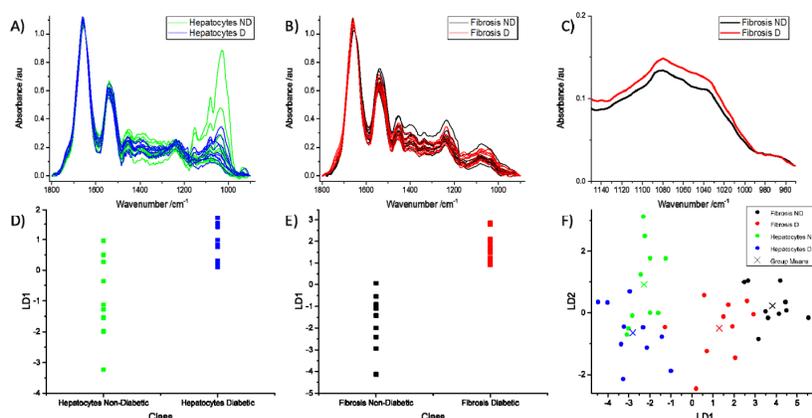

Fig. 4. A) Average spectra from regions of hepatocytes from both diabetic and nondiabetic patients. B) Average spectra from regions of fibrosis from both diabetic and nondiabetic patients. C) Averaged spectra of all fibrotic regions across all diabetic patients compared to same for all non-diabetic patients, along a reduced spectral range. D) Separation by PCA-LDA between diabetic and nondiabetic patients based on hepatocyte spectral characteristics shows overlap. E) Separation by PCA-LDA between diabetic and nondiabetic patients based on fibrosis spectral characteristics has no overlap. F) Averaged spectra form into four distinct clusters based on diabetic status and whether spectra were derived from hepatocytes or from areas of fibrosis.

For further analysis, the patient disease state was diagnosed by a pathologist based on the H&E stained tissue sections. Of the 17 patient cores that could be reliably diagnosed, 11 (6 diabetic and 5 non-diabetic) were grouped as having low-grade liver disease (either non-dysplastic cirrhosis or large cell changes characteristic of low-grade dysplasia) and 6 (3 diabetic and 3 non-diabetic) were grouped as having high-grade liver disease (small cell change characteristic of high-grade dysplasia or HCC). Spectra were extracted from the hepatocyte-associated pixels (the cells conventionally used for the diagnosis) and classified using PCA-LDA, demonstrating a high degree of separation (Fig. 5(A)). Next, from a smaller subset of 3 non-diabetic patients with low-grade disease and 3 non-diabetic patients with high-grade disease, spectra were extracted from the infiltrating fibrosis adjacent to diseased hepatocytes, both directly adjacent to the hepatocytes (within 3 pixels) and distal (over 5 pixels away). These spectra were then analyzed using the unsupervised approach of PCA. Separation between the low-grade and high-grades could be achieved even though the fibrosis is conventionally not used for diagnosis (Fig. 5(B)). This demonstrated that the fibrosis was

biochemically altered in higher grades of liver disease. Interestingly, the distance from the hepatocytes also did not appear to matter and discrimination could be achieved from both adjacent and distal fibrosis pixels, though the adjacent points clustered more tightly (Fig. 5(B)).
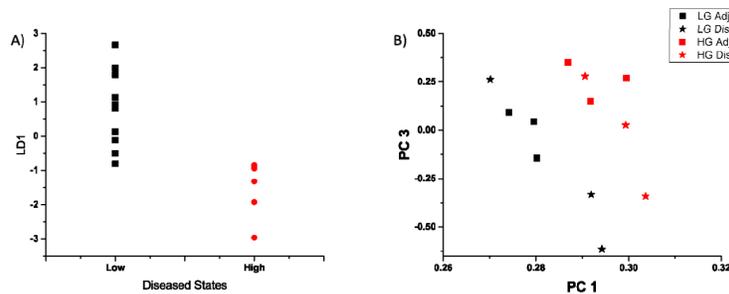

Fig. 5. A) Separation by PCA-LDA was able to distinguish low-grade and high-grade disease in patients on the basis of hepatocyte spectra regardless of diabetic status. B) PCA-LDA was also able to separate low-grade and high-grade disease in patients on the basis of spectra from fibrotic regions both adjacent and distal to hepatocytes.

While the number of cases represented was low, this work demonstrated that fibrosis and to a lesser degree hepatocytes are biochemically altered by the diabetic status of a patient, and in a manner detectable through IR spectroscopy; future work can bolster patient numbers by using tissue sets explicitly designed to recruit diabetic patients and excluding confounding disease states. The goal is not to determine a patient's diabetic status (which requires only blood tests) but to assess the damage that has been caused by diabetes and whether that is related to a faster progression of liver disease; after all, diabetes is an established risk factor for HCC [5, 16]. Future studies can explore whether the level of diabetic damage to liver tissue can be observed using only a few discrete spectral frequencies. This would create an advantage for QCL based discrete frequency imaging approaches where they can leverage a greater speed than conventional FTIR approaches.

The work also demonstrated that fibrosis could be an attractive target for biomarker discovery towards improving diagnosis and prediction of outcome in liver disease as it is not as influenced by the same spectrally confounding factors as hepatocytes and is an active part of the disease process that does not exist within normal tissues. In addition, in a smaller study, we have shown discrimination between low-grade and high-grade disease can be achieved using spectra derived from the hepatocytes and also from the fibrosis adjacent to the damage. This potentially demonstrates that the field effect created by the diseased hepatocytes alters the fibrosis which has a different biochemical signature based on the degree of disease.

Ultimately, this work forms the foundation for future studies investigating whether better predictions of patient outcome can be made, whether better diagnoses can be rendered in regard to the type of liver disease (alcoholic vs. nonalcoholic steatohepatitis), and whether high risk patients can be identified based on their diabetic status. With a strong focus emerging in Pharma on developing anti-fibrosis treatments in the liver, there is need for monitoring exactly how the fibrosis is being altered by these strategies. Infrared spectroscopic imaging, with its sensitivity to biochemical differences in fibrotic regions and capacity for high-throughput assessment of tissue samples, is well positioned to serve as a tool to investigate and take advantage of these often-overlooked aspects of liver histopathology.

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