**Toward surface quantification of liver fibrosis progression**

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Toward surface quantification of liver fibrosis progression

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Liver fibrosis is a wound-healing process in response to various toxic injuries and associated with almost all chronic liver diseases. Inflammatory responses and excessive deposition of extracellular matrix (ECM) are often observed with cirrhosis, marking the end stage of fibrosis that is the major causes of morbidity and mortality worldwide. There is increasing evidence that fibrosis is treatable and reversible in its early stages and even cirrhosis can regress in some clinical studies. Therefore, accurate staging of liver fibrosis is of paramount importance to determine the state of disease progression, responses to therapy, and optimization of treatment to direct disease management.

Currently, researchers have reported many noninvasive methods to assess liver fibrosis. Putative serum makers of fibrosis have been evaluated for the assessment with indirect markers, including serum aminotransferase levels, presence of coagulopathy and platelet counts that reflect alterations in hepatic functions, and markers including laminin, cytokines, collagens, matrix metalloproteinase, and tissue inhibitors of metalloproteinases that reflect serum ECM deposition and turnover. Although some serum assays have been developed to diagnose significant fibrosis and cirrhosis with accuracies, none of them has been validated as a surrogate marker to stage fibrosis due to low sensitivity or nonspecificity. Radiological imaging and novel imaging modalities such as ultrasonography, transient elastography, and magnetic resonance elastography which are based on liver stiffness measurements (LSM), have been shown to be reliable tools to assess liver fibrosis with quick and good reproducibility, but the sensitivity is also less satisfactory with current studies.

Percutaneous liver biopsy still represents the gold standard for the diagnosis and assessment of liver fibrosis. However, the potential complications following liver biopsy and inherent drawbacks, such as the invasive nature of the procedure, sampling error, and inter- and intraobserver variability in the interpretation of the needle biopsy results, have discouraged routine practice. In addition, it is impractical to perform serial liver biopsies to accurately determine the changes of disease progression or to monitor the treatment effects.

To specifically and accurately monitor liver fibrosis in a quantitative manner, information extracted has to be directly compared to morphological features recognized by histological examination of liver biopsy samples. Imaging on the liver surface, especially over a larger sampling area than from biopsy, would therefore give us the potential to extract enough information over a long period of time without the complication brought forth by the invasive biopsy. Laparoscopy has been attempted to diagnose cirrhosis by surface scanning to detect the presence of nodules and the hardening of the tissue. However, with the thick layer of capsule around the liver organ, traditional imaging technology can only focus on the capsule layer without penetrating into the tissue, making it difficult for laparoscopy to extract quantitative information based on histological information that is comparable to biopsy samples.

In recent years, second-harmonic generation (SHG) microscopy has been increasingly used to measure structural protein such as neuron, muscle, and collagen in biological samples with special structural properties. As a nonlinear optical process, SHG exhibits intrinsic advantages over conventional fluorescence by requiring no fluorophore presence in tissue; therefore, signals are not affected by dye concentration and photobleaching. Deeper tissue penetration can also be achieved by using an infrared-range excitation source, resulting in less scattering in tissues than that in the visible wavelength range. SHG has been applied for assessing
fibrosis by quantitative measurement of collagen in various organs, including livers. The results obtained using SHG/two-photon-excited fluorescence (TPEF) are standardized, highly reproducible and can monitor the progression and distribution of collagen at all stages of liver fibrosis with agreement with pathological readings.

To incorporate such properties of SHG and TPEF imaging modalities for potential liver surface scanning based on fiber-optic endoscope and to overcome the current drawbacks from laparoscopy, it is important to assess whether the SHG/TPEF-based methods can extract surface information that reflects the whole organ, especially the interior where biopsy samples are usually obtained. The surface distribution of liver fibrosis features also needs to be examined to direct future scanning locations on the liver surface. Therefore, fully automated quantification algorithms were developed in this paper to extract morphology-based features from both the liver surface and interior of the organ. Analysis of these features was performed to compare to the gold standard (histopathological scorings) to validate features and then to study the representativeness of surface area to the liver interior. We observed that liver anterior surfaces on both left and right lobes contain similar information as in the liver interior when imaged and extracted using SHG/TPEF imaging modalities for fibrosis analysis. This establishes the foundation for future development of laparoscopic nonlinear optical methods to serially quantify liver fibrosis on liver surfaces to eventually eliminate the need for invasive liver biopsy.

2 Methods

2.1 Animal and Tissue Sample Preparation

Male Wistar rats at an average weight of 220 g were housed two per cage in the Biological Resource Centre of Biopolis A*STAR with free access to laboratory chow and water in a 12:12-h light/dark schedule. All animal-related experiments were approved by the Institutional Animal Care and Use Committee. Twenty rats were randomly separated into four groups, with five control rats. Bile duct ligation (BDL) of rats was performed under general anesthesia with ketamine (100 mg/kg body weight). A midline abdominal transverse incision was performed after shaving of the abdomen, exposing the liver and intestines. After gently displacing the liver and intestines, the lower end of the bile duct is identified at its insertion into the small intestines and traced up toward the porta, which is then doubly ligated in two areas near the porta with silk sutures (silk 3.0) and transacted between the two ligation points. Abdominal contents were gently rearranged, and the wound was closed with double-layered tissue closure with vicryl sutures. A total of 15 rats were ligated and sacrificed at intervals of 2, 4, and 6 weeks ($n=5$ per week). Five control rats were also sacrificed at week 0.

Cardiac perfusion with 4% paraformaldehyde was performed to flush out blood cells and fix the liver tissue before harvesting. Liver specimens from both the left lateral lobes and right anterior lobes were preserved in paraffin and sectioned with a thickness of 50 μm.
and a 440–460 nm BP filter before entering the PMT. Using the rest of pixels belonging to noncollagen area. Using the area with strong SHG signals and the other representing the distributions, one representing pixels belonging to collagen the image can be modeled as the mixture of two Gaussian was performed to identify collagen areas in SHG channel im-

gation power at the objective lens is Zeiss, Heidelberg, Germany

lected using a condenser objective lens and recorded by a photo-multiplier tube

ephroscopic mirror was used to collect the SHG signal. The SHG signal was col-

lected with 490 nm BP filter. The SHG signal was collected with a condenser (NA=0.55) and filtered by a dia-

phragm and a 440–460 nm BP filter before entering the PMT for detection. With the intrinsic optical sectioning characteristics for the nonlinear optical process, the pinhole function of the confocal microscope was not used. Reflective SHG signals will be collected along the same optical path as TPEF, but with a different BP filter of 390–465 nm.

Flowchart of the feature extraction algorithm. TPEF and SHG two image channels were separated from the same images. The TPEF channel was clustered into three separate masks by intensity difference, namely, bright, dim, and dark. The bright intensity area in the TPEF channel was classified as hepatocytes mask, the dim area was classified as bile duct cell mask and the dark area was classified as vessel mask including outside-tissue space. Collagen mask in the SHG channel was obtained after segmentation was performed on the images. The feature of the total collagen area was then referred to collagen mask and bile duct proliferation area to bile duct cell mask. Multiplying collagen mask and bile duct cell mask yielded the collagen in bile duct area feature. The remnant hepatocytes area feature was defined as clusters of hepatocytes that were surrounded by bile duct cells; therefore, we obtained it by filing holes of the bile duct cell mask, and then multiplying it by hepatocytes mask.

Fig. 2 Flowchart of the feature extraction algorithm. TPEF and SHG two image channels were separated from the same images. The TPEF channel was clustered into three separate masks by intensity difference, namely, bright, dim, and dark. The bright intensity area in the TPEF channel was classified as hepatocytes mask, the dim area was classified as bile duct cell mask and the dark area was classified as vessel mask including outside-tissue space. Collagen mask in the SHG channel was obtained after segmentation was performed on the images. The feature of the total collagen area was then referred to collagen mask and bile duct proliferation area to bile duct cell mask. Multiplying collagen mask and bile duct cell mask yielded the collagen in bile duct area feature. The remnant hepatocytes area feature was defined as clusters of hepatocytes that were surrounded by bile duct cells; therefore, we obtained it by filing holes of the bile duct cell mask, and then multiplying it by hepatocytes mask.

2.4 Image Acquisition and Segmentation

A total of 12 SHG/TPEF images (3072×3072 pixels, ~1.38×1.38 mm) were scanned for each tissue specimen, with four images covering the liver anterior surface area, four images covering the interior region, and four images covering the posterior surface area. Two specimens were extracted from each animal, with one from the left lobe and one from the right lobe of the liver. For all images, an image-segmentation algorithm based on the mixture Gaussian model was performed to identify collagen areas in SHG channel images. It is assumed that the histogram of intensity of pixels in the image can be modeled as the mixture of two Gaussian distributions, one representing pixels belonging to collagen area with strong SHG signals and the other representing the rest of pixels belonging to noncollagen area. Using the expectation–maximization algorithm, the parameters of the Gaussian distributions was estimated. A binary image was generated by applying a value of 1 to all the pixels having intensity that belongs to the Gaussian distribution representing collagen area and value 0 to the rest of the pixels.

2.5 Features Extraction and Quantification

A total of 12 SHG/TPEF images (3072×3072 pixels, ~1.38×1.38 mm) were scanned for each tissue specimen, with four images covering the liver anterior surface area, four images covering the interior region, and four images covering the posterior surface area. Two specimens were extracted from each animal, with one from the left lobe and one from the right lobe of the liver. For all images, an image-segmentation algorithm based on the mixture Gaussian model was performed to identify collagen areas in SHG channel images. It is assumed that the histogram of intensity of pixels in the image can be modeled as the mixture of two Gaussian distributions, one representing pixels belonging to collagen area with strong SHG signals and the other representing the rest of pixels belonging to noncollagen area. Using the expectation–maximization algorithm, the parameters of the Gaussian distributions was estimated. A binary image was generated by applying a value of 1 to all the pixels having intensity that belongs to the Gaussian distribution representing collagen area and value 0 to the rest of the pixels.

On the liver surface, the tissue edge was detected from the TPEF channel image using the Canny method, which finds edges by looking for local maxima of the gradient in the image. The percentage of collagen of each line, which is parallel to the edge, is calculated. The width of the capsule, which is the unique feature on surface, is defined as the depth where the percentage of collagen changes most between neighboring lines.

Four common features on both the surface (subcapsular region) and in the interior of the liver tissues were quantified, namely, total collagen area percentage, bile duct proliferation area percentage, collagen in bile duct area percentage, and remnant hepatocytes area percentage (Fig. 2). Collagen area percentage is defined as the number of pixels that belongs to collagen in the subcapsular region divided by the total area of the subcapsular region. The segmentation of collagen in the SHG channel image is the same as what is used for capsule identification. Bile duct proliferation area percentage is defined as the number of pixels that belongs to bile duct cells in the subcapsular region divided by the total area of the subcapsular region. Pixels of the TPEF image were clustered into three different groups depending on pixel intensity: completely dark, dim, and bright, which represent areas of the vessel or outside-tissue space, bile duct proliferation area, and hepatocytes, respectively. The clustering was performed by a fuzzy-c-means clustering method. In fuzzy clustering, each point has a degree of belonging to clusters rather than belonging completely to just one cluster and associated with
3 Results and Discussions

3.1 SHG/TPEF Imaging: A Good Substitute for Conventional Histological Imaging

To explore the surface information in the liver, a perfused liver organ was harvested and tissue samples were extracted to expose the liver surface shown in Fig. 3(a). Liver samples were then sectioned in the direction perpendicular to the surfaces in order to keep the capsule collagen for imaging purposes, as shown in the inset of Fig. 3(a). To validate the use of SHG and TPEF microscopy to quantify morphological changes during fibrosis progression, feature compatibility in both the typical histopathological sections and in SHG/TPEF images was investigated. A histopathological tissue section from a fibrotic liver stained with MT [Fig. 3(b)] is compared to a staining-free SHG/TPEF image [Fig. 3(c)] from the same location with the pseudo-colors of green representing SHG signals and red representing TPEF signals. When compared to the stained image and with the major constituents marked, we observed that both the fibril collagen presented in the internal organ and the capsule collagen in the liver surface can be correctly detected by SHG signals. Because of the presence of intrinsic molecules such as NAD(P)H and flavins, which emit substantial fluorescence after excited with femtosecond lasers,

hepatocytes were easily seen as bright red cells in the SHG/TPEF image [Fig. 3(e)]. Bile duct epithelial cells were shown in Fig. 3(c) as the dim red cells due to their lack of fluorescent molecules. The colocalization of these major features suggests that the pathological development of fibrosis on both the surface and in the interior of liver can be investigated by SHG/TPEF imaging for further quantification.

3.2 Defining Capsule and Subcapsule Regions on the Liver Surface

In order to perform a quantitative analysis of liver surface features, we need to define the liver surface with predominantly capillary collagen and the subcapsular region with cells. First, we rotated all images that we obtained into a top empty position whereby there is no tissue sample from the top edge of the image and the liver surface is exposed on the top. Then, combined SHG/TPEF images [Fig. 4(a)] were separated into TPEF [Fig. 4(b)] and SHG [Fig. 4(c)] channels to differentiate the autofluorescence of liver cells from the second harmonic signals of type I collagen. In the TPEF images, the liver boundary was defined as the line boundary between the empty region and the region where there is autofluorescence, and the liver surface is the area below boundary. Because of the morphological difference between the capsule and other liver cells, we separate the surface area into two regions: the capsule and subcapsule regions. The definition of the two was defined in second-harmonic images [Fig. 4(e)]. The SHG image was first converted into a binary image with regions that do not have signals shown as 0 and those with signals as 1. As the line boundary obtained from the red channel was moved pixel by pixel inward, we observed an increase in the average amount of collagen [line average in Fig.
With capsule and subcapsule regions defined, we proceed to extract features from the two regions on the surface and in the liver interior. The defined thickness of the capsule region is a unique feature in the surface area. In the subcapsule region of the surface area and in the interior, the parameters included in the study were total collagen, collagen in bile duct areas, bile duct proliferation, and areas occupied by remnant hepatocytes. The feature extraction methods are elaborated in Section 2, and features extracted have been exemplified as binary maps in Fig. 5 based on the same sample image in Fig. 4. Total collagen [Fig. 5(a)] is defined as the percentage of collagen that was presented in SHG images in the sub-capsule and interior area. Bile duct proliferation [Fig. 5(b)] was defined as the percentage of areas in either the subcapsule or the interior that was occupied by bile duct cells. By overlapping Figs. 5(a) and 5(b), we obtained Fig. 5(c), which is the percentage of abnormal collagen that was distributed in the bile duct area. The area occupied by remnant hepatocytes [Fig. 5(d)] is the percentage of areas taken by remnant hepatocytes, defined as small surviving hepatocyte clusters that are surrounded by bile duct cells, in the subcapsule or liver interior. All four features extracted here were based on comparison to the originally stained tissue samples and the pathologist readings.

### 3.4 Correlation between Liver Surface and Interior

To use the features extracted earlier to establish the relationship between the liver surface and interior in fibrosis progression, we first tested the applicability of our features in assessing fibrosis in the liver interior, where most current studies were focused. Results [Fig. 6(a)] showed that, with the natural progression of liver fibrosis (weeks 0, 2, 4, and 6 after BDL), total collagen, collagen in bile duct areas, and bile duct proliferation increased significantly across different time points ($p < 0.05$). The dramatic increase of bile duct areas in liver interior...
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Fig. 6 (a) Comparison of liver surface to interior. In the liver interior, the percentage areas occupied by four different features was shown at different time points after bile duct ligation. All extracted features show a significant (p<0.05) upward trend with the fibrosis progression except for remnant hepatocytes between week 4 and week 6. (b) The correlation coefficient of features between the liver interior and surface is shown. Features of total collagen, collagen in bile duct, and bile duct proliferation show an upward trend correlation between surface and interior with the plateau at 20-μm depth in the subcapsule region. Remnant hepatocytes have a lower correlation.

3.5 Uniform Fibrosis Distribution across the Anterior Liver Surface

Studies thus far have been performed on the anterior surface of both the left and right lobes of liver tissue. To understand the relationship between different lobes in order to direct future sampling locations, we calculated the correlation coefficient of two lobes based on the four features at different depths of the subcapsule region. The results [Fig. 7(a)] showed that high feature correlation between the two lobes exists and saturates at 20 μm beneath liver capsule for three quantitative features of total collagen, collagen in bile duct areas, and bile duct proliferation. Thus, we can confidently monitor the progress of fibrosis with either lobe by scanning across their surfaces with no misrepresentation of the extent of fibrosis.

All the quantitative analysis was performed on images from the anterior surface of liver because the scanning of liver surfaces would naturally be performed on the anterior of each lobe. In order to understand the fibrosis progression in a global perspective, we also investigated the correlation between anterior and posterior liver fibrosis. Figure 7(b) showed that lower correlation existed between the anterior and posterior liver surfaces based on pathological features. The correlation between the posterior and interior is also weak (data not shown). This indicated that liver fibrosis progresses in a heterogeneous manner toward anterior and posterior surfaces, with anterior surfaces possessing characteristics similar to the interior. Because the anterior surface is the main focus for surface-scanning purposes, it is less important that there are no highly correlated anterior and posterior surface scans.

3.6 Features on or near the Liver Surface are a Good Indication of Liver Fibrosis

Because the liver surface is highly correlated with the interior with the morphological features stated above and the fibrosis distribution is relatively uniform across the liver anterior surface, we focused on the anterior liver surface to quantitatively assess liver fibrosis. A sub-capsule region of 20 μm was shown as an example here because it represents the minimal sampling amount for the meaningful correlation of surface and interior. With a 50-μm thickness of the tissue slices and scanning length of 1380 μm on the anterior surface, an area of 69,000 μm² on the liver surface was covered by each SHG/TPEF image, which is smaller than the equivalent surface area of 225,625 μm², a nonlinear endomicroscopy can scan. The same four features [Fig. 8(a)] were extracted in the subcapsule region, with each quantified at different time points (week 0, 2, 4, and 6 after BDL). Conventional assess-
Fibrosis was scored in five stages, 0, 1, 2, 3, 4, with stage 0 denoting no fibrosis and stage 4 denoting the most advanced fibrosis or cirrhosis. In the subcapsule region, features of total collagen, collagen in bile duct areas, and bile duct proliferation have close agreement with the histopathological scores. The differences between different time points are also significant except for total collagen in early stages between weeks 0 and 2. Areas occupied by remnant hepatocytes, as a less-correlated feature between surface and interior, did not show an observable trend with the progression of fibrosis on the surface; however, just as in the interior, remnant hepatocytes on the liver surface can be a good indicator for the occurrence of fibrosis.

Buschmann and Ryoo\cite{63} suggested that capsular collagen width could be used as a measurement of fibrosis, along with the capsule thickening in liver disease. Here, we quantified the capsule collagen width based on our capsule definition as a unique feature in the surface area. The trend of increasing thickness with fibrogenesis in our model confirmed the nonquantitative findings in the previous study.\cite{63,64} However, we cannot make a clear distinction between each consecutive stage based on statistically significant analysis of this feature even though we can differentiate between the early and late stages of BDL. Therefore, quantification of liver fibrosis on the liver surface works well when SHG and TPEF microscopy enables the quantitative imaging of liver tissue beneath the collagen capsule.

### 3.7 Application in Liver Surface Scanning

With the anterior liver surface containing the similar features as in the liver interior, we compared the normal and fibrotic anterior liver surface by scanning on the front size of the liver. We observed that the correlation coefficients of the left and right liver lobe surface features are shown. Except for the feature of remnant hepatocytes that are less correlated, all other surface features have high correlations reaching a plateau at the subcapsule depth of 20 μm. The correlation coefficient of the anterior and posterior surface features remains at low level, indicating less correlation between these two surfaces.

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**Fig. 7** Quantification of the fibrosis distribution on the liver surface. (a) The correlation coefficients of the left and right liver lobe surface features are shown. Except for the feature of remnant hepatocytes that are less correlated, all other surface features have high correlations reaching a plateau at the subcapsule depth of 20 μm. (b) The correlation coefficient of the anterior and posterior surface features remains at low level, indicating less correlation between these two surfaces.

**Fig. 8** Quantification of liver fibrosis progression on the liver surface. (a) Quantification results of different features extracted from SHG/TPEF images obtained at different time points after BDL showed an upward trend with the fibrosis progression in all features. Significant differences among different time points exist for collagen in the bile duct area and bile duct proliferation in the subcapsule region. This upward trend also agrees with the histopathology scoring results of the same stained tissue samples. (b) Capsule width in the capsule region also increases with the fibrosis progression, with more significant increases in the late stage of fibrosis.
perfused liver using reflective mode SHG and TPEF (Fig. 9). In the capsule region in normal liver [Fig. 9(a)], capsule collagen fibers or fibrous bundles are dense with curry subfilament structures [Fig. 9(c)]. In fibrotic liver, the capillary collagen fibers or fibrous bundles are less dense and straight with loss of curry subfilament structures, likely due to the expansion force in liver (portal hypertension and venopathy, which cause an increase in liver stiffness and cirrhosis). And 20 μm beneath the capsule region in the subcapsule region, normal liver [Fig. 9(b)] has well-organized hepatocytes and no clear collagen presence. While in the BDL fibrotic liver [Fig. 9(d)], bile duct proliferation is obvious that destroys some functional hepatocytes. Abnormal collagen is also present around the bile duct cells, which is a clear indication of fibrosis. Most features are consistent in both the reflective- and transmission-mode images, although the SHG and TPEF signals are weaker in the reflective-mode images. Significant signals can be collected up to 30 μm for SHG and 40 μm for TPEF signals beneath the capsular surface, which is deeper than the statistically meaningful depth of 20 μm to differentiate the normal and fibrotic livers.

4 Conclusions

We discovered a strong correlation between liver fibrosis progression on the anterior surface and the liver interior based on quantitative analysis of morphological features in both regions. Using SHG and TPEF microscopy, we demonstrated the feasibility of monitoring liver fibrosis progression on the anterior surface with the BDL animal model by comparing to the conventional histopathological scoring system. We also discovered a uniform distribution of quantitative liver fibrotic features, such as total collagen distribution, bile duct proliferation, and collagen in bile duct areas, across two main lobes of the anterior liver surface, which gave us confidence to quantitatively monitor the progress of liver fibrosis on different lobe surfaces. We are now one step closer to applying reflective or laparoscopic imaging of the liver surface to stage liver fibrosis, which can potentially be used clinically to complement or eventually replace the more invasive liver biopsy.

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