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NEEDLE-BASED CONFOCAL LASER ENDOMICROSCOPY TO ASSESS LIVER HISTOLOGY *IN VIVO*

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Abstract

Background—Confocal endomicroscopy enables histopathology of the gastrointestinal lumen to be observed *in vivo*. Recent prototypes of a confocal miniprobe are thin enough to be introduced through a needle.

Objective—To evaluate the ability of needle-based Confocal Laser Endomicroscopy (nCLE) to distinguish between normal and cirrhotic liver *in vivo* in a rat model.

Design—Feasibility study.

Setting—Nonsurvival animal experiments.

Interventions—Three normal control and four cirrhotic rats were examined under general anesthesia. The liver was exposed by laparotomy and was imaged using three different prototypes of confocal miniprobes, with 0, 50, and 100 μ m working distance. Images were acquired on the surface of the liver capsule and through a 19-gauge needle inserted into the liver parenchyma. Real-time sequences were recorded after intravenous injection of fluorescein. Biopsy specimens were taken for standard histopathology.

Main Outcome Measurements—Confocal microscopic images of the surface and interior of normal and cirrhotic rats.

Results—Miniprobes with 50 or 100 μ m working distance identified cords of hepatocytes radiating towards central venules in normal livers, and distorted hepatic architecture in cirrhotic livers, either when placed on the liver capsule or when inserted into the parenchyma. The miniprobe with a 0 μ m working distance identified a novel reticular pattern on the liver surface that was detected only in cirrhosis. Like the two longer working distance probes, this probe also identified cords of hepatocytes radiating towards central venules in normal livers, and distorted hepatic architecture in cirrhotic livers, but only when inserted into the parenchyma.

Limitations—Data were assessed in an experimental animal setting, confocal imaging was performed invasively during laparotomy, only one model of cirrhosis was used, and no non-cirrhotic liver diseases were examined.

Conclusions—nCLE provides sufficient detail to distinguish normal from cirrhotic liver in a rat model. This innovative, minimally invasive technique has the potential to provide real-time identification of liver histology during EUS or NOTES procedures.

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INTRODUCTION AND BACKGROUND

Confocal endomicroscopy provides virtual biopsies of tissues so that histological features of the gastrointestinal lumen can be assessed during the course of an endoscopic procedure. Initial studies in patients used this technique to examine dysplasia and neoplasia in colon polyps¹. Subsequent studies have examined a wide range of gastrointestinal conditions, including ulcerative colitis, celiac disease, *H. pylori* infection, gastric cancer, esophageal reflux and Barrett's esophagus, and biliary strictures²⁻⁷. The two types of approaches currently available for confocal endomicroscopy include endoscope-based confocal laser endomicroscopy (eCLE), in which the confocal imaging unit is incorporated into the tip of the endoscope, and probe-based confocal laser endomicroscopy (pCLE), in which the confocal imaging unit uses a probe that is passed through the working channel of the endoscope. A new generation of confocal imaging probes, which has been referred to as needle-based confocal laser endomicroscopy (nCLE), are now being developed that are narrow enough to fit through a needle. Availability of such probes has the potential to permit virtual biopsies in real time of solid organs and other intraperitoneal structures that are accessible during laparoscopy, EUS, or NOTES procedures. Indeed, one proof-of-principal study used an earlier prototype of an nCLE probe to collect images from various abdominal organs in pigs undergoing NOTES and EUS with FNA⁸, while a preliminary report used the current generation of nCLE probes to describe the interior of pancreatic masses and cysts in patients undergoing EUS with FNA⁹.

Histological assessment is a particularly important diagnostic tool in liver disease. Liver biopsies are followed for grading and staging of patients undergoing treatment for chronic viral hepatitis, and are used to establish the diagnosis and determine the extent of fibrosis in a number of other types of liver disease as well. It has been estimated that as many as 3% of liver biopsies result in complications, which can include pain, bleeding, bile peritonitis, and pneumothorax^{10, 11}. Therefore, the ability to obtain directed virtual biopsies of the liver in a minimally invasive fashion could have a significant clinical impact. Liver biopsies already are obtained during laparoscopy or by guidance of external or EUS imaging¹², so nCLE imaging of the liver could be incorporated into these procedures to confirm that an intrahepatic location of a biopsy site is appropriate or to obtain immediate histological results. The purpose of this study was to test three available prototype nCLE probes with different working distances to determine whether they are adequate for determining the presence of liver disease and to compare their relative efficacy.

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 250–300 g were used for all experiments. Common bile duct ligation was performed as described previously¹³, and animals were studied two weeks after surgery. Three normal control animals and four animals that underwent bile duct ligation were studied. Animals were housed in temperature- and light-controlled rooms and maintained on a standard diet prior to study. All studies were approved by the Yale Animal Care and Use Committee. Sodium fluorescein was purchased from Akorn, Inc (Lake Forest, IL).

Confocal imaging

Animals were anesthetized with isoflurane anesthesia, and then the liver was exposed by a midline laparotomy. Fluorescein (0.25 ml of a 1.0% solution in saline) was injected into the inferior vena cava, and then confocal imaging was performed 5 min after injection of the dye. Three confocal probes were used to examine each liver. nCLE probes were used to

examine rat livers in live animals during laparotomy. The probes were designed to observe focal planes that are a short (0 μm), medium (50 μm), or long (100 μm) distance from the tip of the probe, and each probe fit through the lumen of a 19 gauge needle (Figure 1). Each probe had a lateral spatial resolution of 3.5 μm , and a field of view of 320 μm . The probes differed in terms of the plane of focus each one detected; one probe focused on tissue 0 μm from the tip of the probe, the second one focused 50 μm from the tip, and the third focused 100 μm from the tip. The 0 μm and 100 μm working distance probes were specially designed prototypes with a diameter of 0.85 mm and a standard CholangioFlex probe (diameter 0.94 mm) was used for 50 μm -deep imaging. All three confocal probes were used with a Cellvizio confocal system (Mauna Kea Technologies, Paris, France). Hepatic fluorescein was excited using the 488 nm line of an argon laser, and fluorescence emission >515 nm was collected. For each probe, up to one minute of images was collected with the probe gently placed on the surface of the liver, then a 19 gauge needle with a stylus was inserted into the hepatic parenchyma, the stylus was removed, and the probe was inserted through the barrel of the needle to collect images of the interior of the liver as well. H&E sections of liver were obtained from normal control (Figure 2a) and bile duct-ligated (Figure 2b) animals to confirm normal and cirrhotic histology, respectively. For each probe, images were collected first by placing the probe in gentle contact with the liver capsule (Figure 3), and then a 19 gauge needle with a stylus was inserted into the hepatic parenchyma, the stylus was removed, and the probe was advanced through the lumen of the needle to the liver parenchyma where additional images were obtained (Figure 4). Animals were euthanized by exsanguination at the conclusion of the study.

Multiphoton imaging

Normal and two week bile duct obstructed rats were used. Livers were snap-frozen in liquid nitrogen-cooled freon to reduce freezing artifact. Prior to imaging, the tissue was thawed in 4% formalin in 0.1M PBS and fixed for 1 hr at room temperature. The liver pieces were transferred to a glass coverslip with the capsule facing the objective lens. The liver pieces were examined using a Zeiss LSM 510 NLO microscope equipped with a SpectraPhysics MaiTai fsec-pulsed multiphoton laser (Mountain View, CA). Specimens were scanned with an excitation wavelength of 735 nm and fluorescence images were collected using non-descanned external detectors in two channels, 320–380 nm to detect second harmonic generation (SHG) and 410–490 nm to detect tissue autofluorescence. These excitation and emission wavelengths were chosen because they are optimal for examining tissues of the digestive system¹⁴.

RESULTS

Normal liver

For images acquired at the liver capsule, both the medium (Figure 3b) and long (Figure 3c) working distance probes revealed typical hepatic architecture, including cords of hepatocytes radiating towards central venules that were evident on H&E sections subsequently prepared from the same livers (Figure 2a). Although hepatocytes also were observed using the short working distance probe, no typical organized cord pattern was appreciated (Figure 3a).

Cirrhotic liver

Liver cirrhosis was induced by common bile duct ligation (BDL), and then livers were examined two weeks later. This is a widely used model of cirrhosis that induces a portal-based fibrotic pattern¹³. As with control animals, images were collected first by placing each probe in gentle contact with the liver capsule (Figure 5), and then a 19 gauge needle with a stylus was inserted into the hepatic parenchyma, the stylus was removed, and the

probe was advanced through the lumen of the needle to the liver parenchyma where additional images were obtained (Figure 6). Normal liver architecture was not seen under any circumstances. For images acquired at the liver capsule, both the medium (Figure 5b) and long (Figure 5c) working distance probes revealed regions of cells that were surrounded by broad, irregular white bands. Although the exact histological correlate of this could not be confirmed, the pattern is suggestive of the fibrotic pattern observed in H&E sections of the same tissue (Figure 2b). In contrast, the short working distance probe unexpectedly revealed a diffuse reticular pattern with no evident cells in the plane of focus (Figure 5a). For images acquired within the hepatic parenchyma, similar patterns were observed with all three probes, which showed regions of cells irregularly bounded by bright bands (Figure 6). A mosaic reconstruction in which adjacent images were overlaid (Figure 7) suggested the similarity in this pattern to the fibrotic pattern seen in H&E sections (Figure 2b), although such reconstructions typically are intended to stitch together images collected in two rather than three dimensions. Because of the dramatic change in the appearance of the liver capsule seen in cirrhosis when using the shallow working distance probe, H&E images of the capsule were compared in control and BDL specimens (Figure 8). The mesothelial cell layer covering the liver was significantly thickened in BDL relative to control liver. Next, the region of the liver capsule was fixed but unstained and then examined by multiphoton microscopy (Figure 9). Cellular structures could be identified by autofluorescence, while extracellular collagen was identified by SHG. A thin layer of collagen overlaid the outermost hepatocytes in both control (Figure 9a) and BDL (Figure 9b) livers. However, in BDL livers, a delicate network of autofluorescence elements overlaid the collagen, which corresponds with the location of the mesothelial cells comprising the liver capsule.

DISCUSSION

The current work suggests that nCLE can be used to detect the histological architecture of normal liver. For images acquired within the hepatic parenchyma, typical hepatic architecture was detected using each of the three probes (Figure 4). The medium and long working distance probes appear similarly useful regardless of whether they are inserted into the hepatic parenchyma, or are used less invasively by merely being placed in contact with the liver. In contrast, the short working distance probe reveals typical hepatic histology only when placed into the interior of the liver. This may reflect the fact that the lobular structure of the liver is less well defined in the region just beneath the liver capsule, and so might not be readily detected if the depth of focus is too shallow. The current work also suggests that nCLE can be used to detect the histological architecture of cirrhotic liver. The medium and long working distance probes appear to give similar information regardless of whether they are inserted into the hepatic parenchyma, or are used less invasively by merely being placed in contact with the liver. In contrast, the short working distance probe appears to reveal a novel change in structure of the liver capsule seen in this animal model of cirrhosis when used minimally invasively by merely being placed in contact with the liver. Although this cell layer has not been characterized in detail, it is well known that hepatic stellate cells become activated in cirrhosis, and adopt a myofibroblastic phenotype that includes increased expression of actin^{15–17}. Therefore it is possible that the cell layer comprising the liver capsule undergoes a similar phenotypic change.

Confocal fluorescence microscopy is a microscopic technique that permits fluorescence in cells and tissues to be detected within a narrow plane of focus. This in turn permits cellular and even subcellular structures to be distinguished within thick tissue preparations^{18, 19}. This technique has become a widely used basic research tool over the past twenty years. Common applications have included localization of immunolabeled proteins in fixed cells and tissues, detection of fluorescent dyes to monitor subcellular changes in calcium and pH in live cells and tissues, and use of green fluorescent protein and its variants in live cells to

monitor trafficking and subcellular distribution of fluorescently tagged proteins. More recently, advances in optics and microscopy have permitted confocal imaging to be combined with endoscopy to permit confocal microscopy to be performed during the course of standard endoscopic procedures. Fluorescein is the principal dye that has been used for confocal endomicroscopy, in part because of its fluorescence properties and safety profile²⁰. This has allowed some of the key histological properties of the gastrointestinal tract to be observed in real time^{1, 4}, and has even allowed development of novel functional tests of gastrointestinal physiology^{21, 22}. Initial studies showed that the diagnostic yield of confocal endomicroscopic images of colonic polyps was comparable to the diagnosis obtained by examining the resected polyps by standard H&E techniques¹. More recent studies have tried to go beyond showing that confocal endomicroscopy is as effective a diagnostic tool as examining endoscopic biopsy specimens. Multicenter trials now are testing whether the diagnostic yield is increased when confocal endomicroscopy is used in procedures such as screening Barrett's tissue for dysplasia²³ or looking for cholangiocarcinoma in indeterminate biliary strictures²⁴. The next frontier for real time assessment of histology in vivo may be to examine extraluminal organs such as the pancreas and liver, and the development of confocal probes that fit within the shaft of a needle make this possible for the first time. A preliminary report suggests that cellular elements within pancreatic masses can be identified by nCLE when the probe is inserted into the mass through an EUS-guided FNA needle⁹. There have been two other reports of confocal imaging of the liver. One report used a rigid confocal probe to examine the livers of patients, and steatosis and fibrosis could be detected²⁵. The other report used a flexible confocal probe to examine pig livers during NOTES procedures, but that probe was a forerunner of the ones used in this study and was too fragile to be used in humans. Moreover, limited histological detail was provided⁸. The liver is accessible via an EUS-guided FNA needle¹², and the current work provides the first evidence that nCLE examination of the liver via that approach may provide useful histological information. Because the liver is the principal route for collecting and eliminating organic anions such as fluorescein from the bloodstream, hepatocyte fluorescence following injection of fluorescein is much more intense than fluorescence in the blood, so blood in the hepatic parenchyma does not interfere with nCLE imaging of hepatocyte fluorescence. Rats were used in this study because this animal model has been characterized most extensively and is thought to closely approximate the structure and function of human liver²⁶. A particularly novel finding of the current study was that normal and cirrhotic liver could be readily distinguished from each other by placing a 0 μ m working distance probe on the liver surface. If substantiated, such an approach has the potential to minimize the risk of bleeding from a liver biopsy by obviating the need to penetrate the liver with a needle. However, it is not yet clear whether these microscopic changes in the liver capsule are seen only in cirrhosis, or can be seen in pre-cirrhotic stages of fibrosis as well. If so, then nCLE would offer information beyond what could be obtained through modalities such as simple laparoscopic or NOTES examination of the liver. There are several limitations to the current observations, including that data were assessed in an experimental animal setting, confocal imaging was performed invasively during laparotomy, only one model of cirrhosis was used, and no non-cirrhotic liver diseases were examined. Finally, like pCLE but in contrast to eCLE, nCLE does not permit the focal distance to be modulated. As a result, nCLE imaging can not be used to reconstruct three-dimensional information about tissue structure. Further studies will be needed to define the role of nCLE in the diagnosis of liver disease in patients.

Acknowledgments

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ACRONYMS

BDL	bile duct ligation
EUS	endoscopic ultrasound
eCLE	endoscope-based confocal laser endomicroscopy
FNA	fine-needle aspirate
H&E	hematoxylin and eosin
nCLE	needle-based confocal laser endomicroscopy
NOTES	natural orifice transluminal endoscopic surgery
PBS	phosphate-buffered saline
pCLE	probe-based confocal laser endomicroscopy
SHG	second harmonic generation

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Figure 1. A probe used for needle-based confocal laser endomicroscopy (nCLE)
Image shown is of the needle probe with a 0 μm working distance protruding from a 19 gauge needle.

Figure 2. Hematoxylin and Eosin-stained sections of liver from normal and cirrhotic rats
(a) Normal rat liver. Cords of hepatocytes radiating towards a central venule can be appreciated. Image was obtained using a 10 $\times$ objective lens (scale bar, 100 μ m). **(b) Cirrhotic rat liver.** Image was obtained two weeks after ligation of the common bile duct. Nodules of hepatocytes are surrounded by fibrous bands that include proliferating bile ductules. Image was obtained using a 4 $\times$ objective lens (scale bar, 250 μ m).

Figure 3. nCLE images of normal liver acquired at the liver capsule
(a) 0 μm working distance. Individual hepatocytes can be seen (arrows) but no regular pattern is observed. **(b) 50 μm working distance.** Cords of hepatocytes radiating towards a central venule are observed. **(c) 100 μm working distance.** Cords of hepatocytes radiating towards a central venule are observed. Results shown here and in the next figure are representative of what was observed in each of three control animals.

Figure 4. nCLE images of normal liver acquired from the hepatic interior (a) 0 μm working distance; (b) 50 μm working distance; and (c) 100 μm working distance. In each case, cords of hepatocytes radiating towards a central venule are observed.

Figure 5. nCLE images of cirrhotic liver acquired at the liver capsule

(a) 0 μm working distance. A coarse, diffuse reticular pattern is observed. No individual cells are appreciated. **(b) 50 μm working distance.** Clumps of cells separated by bright bands are observed. **(c) 100 μm working distance.** Clumps of cells separated by bright bands again are observed. Results shown here and in the next figure are representative of what was observed in each of four bile duct-ligated animals.

Figure 6. nCLE images of cirrhotic liver acquired from the hepatic interior (a) 0 μm working distance; (b) 50 μm working distance; and (c) 100 μm working distance. In each case, clumps of cells sometimes separated by bright bands are observed.

Figure 7. Mosaic reconstruction of adjacent confocal images in a cirrhotic liver

View of adjacent images stitched together shows that the pattern of bright bands (denoted by arrows) surrounding clusters of cells reflects the size and shape of the fibrous bands that separate cirrhotic nodules in this animal model. The 100 μm working distance probe was used to collect these images.

Figure 8. Comparison of the liver capsule under normal conditions and after bile duct ligation
(a) Normal liver. The liver edge is covered by a single layer of squamous epithelium (arrow). **(b) Cirrhotic liver.** The epithelial layer covering the liver has increased several-fold in thickness (arrow). Both images were obtained using a 60 $\times$ objective lens (scale bars, 25 μ m).

Figure 9. Multiphoton imaging of the liver capsule under normal conditions and after bile duct ligation

(a) Normal liver. Hepatocytes (green) are detected by autofluorescence, and are covered by a thin layer of collagen (blue) detected by SHG. **(b) Cirrhotic liver.** Hepatocytes again are covered by a thin layer of collagen, but here the collagen then is overlayed by a fine network of additional autofluorescence elements (arrows). The location of this network corresponds to the location of the squamous epithelium comprising the liver capsule (Figure 8b) and the reticular pattern is similar to what is observed by nCLE examination of the capsule (Figure 5a).