Optically clearing tissue as an initial step for 3D imaging of core biopsies to diagnose pancreatic cancer

Ronnie Das*a, Aishwarya Agrawalb, Melissa P. Uptonc, and Eric J. Seibela

aHuman Photonics Laboratory, University of Washington, 4000 Mason Road, Seattle, WA 98195
bIndian Institute of Technology Gandhinagar, Ahmedabad, 382424 India
cUniversity of Washington Medical Center, Pathology Department Box #356100, Seattle, WA 98195

ABSTRACT

The pancreas is a deeply seated organ requiring endoscopically, or radiologically guided biopsies for tissue diagnosis. Current approaches include either fine needle aspiration biopsy (FNA) for cytologic evaluation, or core needle biopsies (CBs), which comprise of tissue cores (L = 1-2 cm, D = 0.4-2.0 mm) for examination by brightfield microscopy. Between procurement and visualization, biospecimens must be processed, sectioned and mounted on glass slides for 2D visualization. Optical information about the native tissue state can be lost with each procedural step and a pathologist cannot appreciate 3D organization from 2D observations of tissue sections 1-8 μm in thickness. Therefore, how might histological disease assessment improve if entire, intact CBs could be imaged in both brightfield and 3D? CBs are mechanically delicate; therefore, a simple device was made to cut intact, simulated CBs (L = 1-2 cm, D = 0.2-0.8 mm) from porcine pancreas. After CBs were laid flat in a chamber, z-stack images at 20x and 40x were acquired through the sample with and without the application of an optical clearing agent (FocusClear®). Intensity of transmitted light increased by 5-15x and islet structures unique to pancreas were clearly visualized 250-300 μm beneath the tissue surface. CBs were then placed in index matching square capillary tubes filled with FocusClear® and a standard optical clearing agent. Brightfield z-stack images were then acquired to present 3D visualization of the CB to the pathologist.

Keywords: 3D pathology, specimen preparation, optical clearing, core biopsy processing.

1. INTRODUCTION

Fewer than 5% of pancreatic cancer patients survive more than 5 years after initial diagnosis1-3. Pancreatic adenocarcinoma incidence and mortality rates are almost the same, reflecting advanced stage at diagnosis and tumor aggressiveness. Recently, this cancer has attracted media attention after claiming several famous people, including first American female astronaut Sally Ride, Apple creator Steve Jobs, opera singer Luciano Pavarotti and actor Patrick Swayze. For current patients and high risk individuals, increased awareness is overshadowed by questions such as “what exactly causes the disease?”, or “what can we do to detect pancreatic cancer sooner?” or “can we develop effective screening methods?” These questions reflect the gap in knowledge concerning the pathology and development of pancreatic adenocarcinoma3-4.

To obtain tissue, fine needle aspirates (FNAs) and core biopsies (CBs) are procured directly from patients under radiological, or endoscopic guidance5-7. FNAs, consisting of aspirated cells, are processed in pathology laboratories, and cells dispersed onto 2D microscopic slides are examined using brightfield optical microscopy. CBs, which are thin cylinders of intact tissue, are similarly observed and assessed, but only after cores are thinly sliced and mounted on slides. Relatively speaking, more information about the disease is obtained when observing tissue sections from CBs than from FNA smears; even more is learned when observing intact CBs instead of tissue slices (since tissue is inherently lost and distorted in sectioning). Therefore, combining optical microscopy traditionally employed in pathology with intact CBs necessitates the ability to image specimens in 3D.
Imaging and visualization of large, intact CBs in 3D using brightfield optical microscopy have several, important advantages. Tissue architecture, morphology and structural features would be preserved and would best represent the native in vivo condition; this feature would also provide more insight on how the pathology presents and would therefore enhance diagnosis. Additionally, utility of CBs would be maximized, since no tissue would be wasted or lost due to processing, or slicing. The intact CB is an extremely valuable specimen as it is often the only tissue obtained and analyzed before making a life-changing decision about suspected pancreatic cancer. Since computing power and memory are no longer a premium, 3D visualization via image reconstruction can also render 2D image slices at any focus plane, or angle, if traditional observations became necessary for the pathologist.

Currently, several imaging modalities are employed to image and visualize biospecimens in 3D. They include the confocal NIR reflectance and two-photon fluorescence laser scanning, optical coherence tomography (OCT), optical projection tomography (OPT) and the most recently developed photoacoustic microscope and the optical projection tomographic microscope (OPTM) (figure 1). Of these techniques, confocal, OCT and OPT and OPTM represent the best imaging modalities for transitioning from 2D to 3D pathology. However, while confocal and OCT can image tissue in 3D with unfixed tissue, the methods preclude the use of traditional histological stains and/or fluorescently-tagged biomarkers which are standards in diagnostics: incorporation into the traditional workflow is paramount to moving 3D pathology and diagnosis forward. In comparison, OPT and OPTM are the closest techniques to brightfield optical microscopy currently employed by clinicians as pathologists rely on transmission mode absorption imaging. OPTM, additionally, can also simultaneously image biospecimens in fluorescence mode if specific fluorescently-tagged biomarkers are needed for a more informed diagnosis.

Brightfield 3D imaging of large CBs via OPTM requires one basic step: the ability for light to transmit through thick, pancreas CBs that are optically and mechanically inhomogeneous. At high magnification, CBs appear dark and opaque on optical microscopes because of the small proportion of ballistic photons, i.e. the majority of photons are absorbed and/or scattered. Light scattering is bar far the greatest obstacle and occurs due to the numerous interfaces and media found in soft tissue; extra- and intracellular fluid, connective tissue, cell and nuclear membranes, cytosol and intracellular organelles contribute to the wide array of refractive indices which result in photon scattering. To this end, optical clearing agents (OCAs) have been used extensively over the last decade (figure 1) to optically clear otherwise opaque specimens by diffusing into the tissue and homogenizing the various refractive indices. Consequently, photon scattering is significantly reduced while the total number of ballistic photons is increased. OCAs have also been shown to be effective in the presence of absorptive stains and vessel paints.

In this study, our multidisciplinary research team has optically cleared pancreatic tissue for the first time in a systematic investigation towards furthering 3D pathology to enhance diagnosis for pancreatic cancer. By employing a newly
developed, pancreas-specific OCA, pancreatic tissue slices and large CBs were optically cleared and imaged both macroscopically and microscopically. Single and z-stack images of tissue at fixed focus planes and multiple planes were recorded for qualitative and quantitative analysis. Cleared CBs were also imaged within enclosed square capillaries at orthogonal perspectives to compare and contrast the different OCAs while taking the first initial steps towards 3D visualization of the entire CB.

2. METHODS

2.1. Pancreas tissue procurement

Porcine pancreas was purchased and obtained from a local vendor (Kapowsin Meats, Inc.) after pathologists advised that porcine pancreas tissue best simulates human pancreas tissue. After manually teasing and removing connective tissue and the peritoneum from the organ, pancreas tissue was fixed for a week in a standard fixative employed in pathology laboratories (10% neutral buffered formalin, HT501128-4L, Sigma-Aldrich, Co.). To ensure homogeneous and complete perfusion of fixative, solution volumes were replaced daily with fresh fixative.

Pancreas tissue slices were produced by a standard microtome (figure 2) through collaborations with the Department of Pathology at the University of Washington Medical Center. Pancreas tissue was sectioned in slices with thicknesses from 40-60 µm. CBs were produced by a custom cutting device consisting of two parallel-positioned microtome blades (Coated High Profile Disposable Blades, #1001593, Thermo Scientific, Inc.). Pancreas tissue was cut into thick sheets; those sheets were subsequently sectioned with the device to produce rectangular prism-shaped CBs 1 cm long and at several thicknesses spanning 0.2-0.8 mm.

2.2. Optical monitoring chamber, capillaries, solution volumes

A custom-made chamber (25 x 10 x 2 mm) was fabricated from microscope slides to contain both tissue slices and CBs. Chamber dimensions permitted solution volumes 50-80x greater than specimen volumes to ensure homogeneous diffusion. A pancreas-specific OCA (FocusClear®, Cell Explorer Labs, Inc.) was employed for optically clearing fixed and standard hematoxlin-stained pancreas specimens. Square quartz (s = 1 mm, QS101-300, VitroCom, Inc.) and borosilicate (s = 2 mm, S102-300, VitroCom, Inc.) capillaries were purchased to best match the refractive indices of both FocusClear® and a common OCA (50/50 PBS/glycerol).

2.3. Optics and image processing

All still images were taken from movie recordings of the experiments conducted. Macroscopic images were taken with a high contrast, high resolution color digital camera (CoolPix L110, Nikon Inc.) and CCD sensor (CFW-1312C, Scion Corporation, Inc.). Microscopic images were obtained with a high contrast gray scale CCD camera (Prosilica GE 1650, Allied Vision Technologies, Inc.) attached to an Olympus inverted microscope with standard 10, 20 and 40x objectives. Diffraction gratings were used to determine spatial resolution (740, 370 and 185 nm/px, respectively). z-Stack images were acquired by precisely moving the microscope objective in steps ranging from 1-10 µm using a piezoelectric motor (NV40/1CL, Piezosystem Jena, Jena, Germany). Steps were determined by the objective's depth-of-focus (10, 6 and 1 µm, respectively). The microscope xy-translation stage was moved in a similar fashion using a stepper motor. All motor
function was controlled via a custom-written LabVIEW program. Corresponding images were acquired and processed using ImageJ and quantitative data were measured using both ImageJ and MATLAB. z-Stack images were recorded across the entire CB when placed in the chamber and square capillaries.

3. RESULTS

3.1. Pancreas tissue slices

3.1.1. Macroscopic observations

To demonstrate the reduction of light scattering by the OCA, pancreas tissue slices were placed in the glass chamber filled with formalin. Specimens were then illuminated with a white light source from the side. In figure 3, the chamber was placed on a black opaque background and a light platform was set upright so that light rays traveled from the right to the left side of figure 3a. In this fashion, the tissue slice was visualized by light rays scattering off the tissue and towards the camera system recording above the chamber. The tissue slice in figure 3 is ~15 mm long, ~10 mm wide and 60 µm thick. After optically clearing for 30 min, the tissue slice was nearly invisible to the naked eye in figure 3d. FocusClear® was then replaced with the original formalin solution and reversibility of the OCA with pancreas specimens was successfully demonstrated.

3.1.2. Microscopic observations

Next, the same experiment was repeated but we imaged pancreas tissue slices microscopically with a 40x objective and using transmissive lighting. The tissue slice in figure 4b was measured to be 20 x 10 x 0.060 mm, yielding an xy-area of 200 mm². The tissue area imaged in figure 4a is 0.66 mm² (~0.3% of total tissue slice area). At 40x, the field-of-view with the CCD camera was 300 x 220 µm (see green rectangle and figure text). Therefore, the imaged areas in figure 4a were produced by taking multiple overlapping pictures across the tissue by using the motor system to shift the xy-translation stage in one-dimension at incremental steps. By using a custom-written image registration and stitching program, we were able to rapidly generate large panoramic images of the tissue slice.

Figure 4a is a comparison of the tissue slice when (upper panorama) uncleared versus (lower panorama) the specimen being optically cleared for 30 min. Red squares throughout the panoramic image represent regions of interest (#1-5), where the intensity of light was measured and compared between the uncleared and cleared conditions. While most of the cleared tissue (on the left hand side) was optically homogeneous, the region of interest at the far-right illustrated pancreas islets that were overlapping each other in the z-dimension. After optical clearing, it became clear (more so through the eyepieces) which islet was on top; other ducts were also able to be visualized in this region.

3.2. Pancreas CBs

3.2.1. Macroscopic observations

As an initial step, a pancreas CB was compared when it was fixed, stained and optically cleared under transmissive lighting. After fixation and cutting, the CB

Figure 3: Image sequence from a movie recording of optically clearing (in a reversible manner) a pancreas tissue slice. a) Tissue slice in glass chamber. b) Micropipette used to manually remove the formalin solution and c) the image immediately following the replacement with FocusClear®. d) Tissue in OCA for 30 min. e) OCA was removed and replaced with previous formalin solution. f) Pancreas tissue in new formalin solution volume after 10 s.
Figure 4: Optically clearing pancreas tissue slice under transmissive lighting.  a) A 40x objective was used to image the 60 µm thick tissue slice in the b) glass chamber before and after optically clearing. The green box outlines the 40x objective field-of-view. 15 such windows were registered and stitched together into one panoramic image using a custom-written algorithm. The repetitive black smudge throughout the panoramic image was debris on the objective, therefore the artifact appeared in every window that was subsequently stitched together. c) Quantitative data of the amount of transmissive light through the specimen before and after optical clearing. Regions of interest are displayed as red squares in 4a from left to right.

was placed in a standard histological stain used in pathology laboratories (hematoxylin) for approximately 10 min and was then optically cleared for an additional 10 min. Images were taken at each subsequent step (figure 5).

3.2.2. Microscopic observations

A pancreatic CB (L = 1.1 cm, w = 500 µm, th = 550-600 µm) was put into the glass chamber and at 20x, a movie was recorded before optical clearing, after the OCA was added and at subsequent time intervals thereafter (figure 6). Initially the CB was opaque (figure 6b). After OCA immersion for 60 min, transmissive light intensity increased from 5-15x. With OCA application to tissue, it is known that osmotic-related effects are increased²⁶. Consequently, the red arrows in figure 6e were used as markers to precisely align corresponding images within the movie since the CB increased in volume by ~20% after being immersed in the OCA for 60 min.

The same experiment was then conducted with a 600 µm thick CB at 40x, except that the objective was raised in 2 µm steps to produce a z-stack of images spanning the entire CB thickness (figure 7). A 2 µm step was chosen since a 40x objective depth-of-focus is ~1 µm. The procedure was then repeated when the OCA was immediately applied (0 min, figure 7a-middle) and after the CB remained immersed in the OCA for a 45 min period (figure 7a-right). Imaging and movement of the xy-translation
stage were done very carefully so that 1) during OCA application, the CB did not shift in position and 2) while immersed in the OCA, the CB remained in a similar position since the tissue appreciably increased in volume (compare figure 7a-left to figure 7a-right).
Figure 7: Optically clearing a pancreatic CB and imaging in a (2 µm incremented) z-stack at 40x. a) From left to right, formalin-fixed CB, and CB in FocusClear® for 0 and 45 min. The image chosen from the z-stack is at the z-plane where the islets (red arrow) are best in focus (250-300 µm below tissue surface). b-d) Regions of the images in a where the islets resided. e-g) Simple edge detection of images 7b-d. e) Initially, no islets were able to be distinguished. f) Approximately 3 islets were able to be seen immediately after adding FocusClear®. g) After 60 min in the OCA, it was discernable that the region contained 6 islets. h) Quantitative data of transmissive light intensity from regions encompassed by dark and light colored purple boxes in 7a.

Three features from this simple experiment were able to be measured and observed. First, transmissive light intensity at specific regions of interest (purple boxes, see figure 7 text) were quantitatively measured and compared between the fixed CB, the CB immersed in the OCA and the CB immersed in the OCA for 45 min. Second, islets buried deep within the CB were able to be visualized with optical clearing that otherwise were not visible in the uncleared condition. Finally, islet structure became clearer with the application of FocusClear® and was qualitatively shown by using a simple edge detection plug-in feature of ImageJ.

Finally, glass capillaries containing CBs were filled with both FocusClear® and 50/50 PBS/glycerol solutions, which is a standard OCA used in many optical clearing applications. z-Stack images of CBs were then recorded in order to view...
islet-containing pancreas CBs while immersed in formalin and the OCA of interest for 10 min and 5 hr. Results of these experiments are summarized in table 1.

Table 1: Application of OCAs on pancreas CBs buried 100-150 µm below the tissue surface. The specific clearing agent and the type of glass capillary used is displayed below. Red text indicates the measured increase in transmissive light within the given islet area in comparison to when the CB was immersed in formalin. The CB at 5 hr for FocusClear® is present, but was completely transparent. However, the red arrows point to an optical artifact on the objective that appeared in both images.

<table>
<thead>
<tr>
<th></th>
<th>PBS (borosilicate)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>formalin</td>
<td>10 min</td>
<td>5 hr</td>
</tr>
<tr>
<td></td>
<td>1.3x</td>
<td></td>
<td>1.8x</td>
</tr>
<tr>
<td></td>
<td>50/50 PBS/glycerol (borosilicate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>1.8-4.5x</td>
<td>NO DATA</td>
</tr>
<tr>
<td></td>
<td>5 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FocusClear® (quartz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>2.4-4.2x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 hr</td>
<td>4.5x</td>
<td></td>
</tr>
</tbody>
</table>

4. DISCUSSION

OPTM may provide the best method to make 3D pathology a reality since the imaging modality employs brightfield illumination and microscopy similar to conventional pathology. However, average CB thickness precludes OPTM since a majority of the light is scattered. Without a significant number of ballistic photons, image projections acquired by OPTM are low quality and result in poor 3D reconstructions and visualizations. Additionally, if enough transmissive light can penetrate the CB, absorptive histological stains will decrease the total number of ballistic photons and again would result in low quality image projections. To this end, our research team has successfully demonstrated that both pancreas tissue slices and CBs can be optically cleared such that the intensity of transmissive light is increased on the order of 5x in thin tissue and 15x in thick tissue compared to background (no tissue). This may be the first time that
quantitative and qualitative optical data were measured for thin pancreas tissue slices and CBs after specimens were cleared using a pancreas-specific OCA (FocusClear®).

We have also shown that optical clearing of pancreas tissue is reversible and allows observations of specific pancreatic structures buried *deep* within the tissue. These two features are very important in that reversibility will allow CBs (to be) imaged in OPTM to be removed from the apparatus and processed via traditional pathology laboratory procedures. Visualization of structures deep within tissue will also contribute to the general understanding of the *in vivo* tissue state, such as tissue architecture, and morphological/structural features (figure 7a-right, 7e-g). An initial exponential fitting of the transmissive light intensity for the CB (figure 6g) demonstrated a diffusion-limited process and that the CB immediately underwent a 4x increase in light intensity after the application of FocusClear®. Although transmissive light reached a maximum at 50% of background light intensity (figure 6f-g), light intensity may vary greatly between samples and may increase altogether if specimens are left unfixed.

In cases where high concentrations (6.5 mM) of hematoxylin was applied that left CBs completely opaque (figure 5-middle), optical clearing permitted enough transmissive light to match 50% of the light intensity that was measured in specimens that were fixed, but unstained. It is expected that hematoxylin stained tissue will absorb a significant number of photons and thus decrease the total number of ballistic photons, thereby reducing the quality of image projections for image reconstructions in OPTM. However, stained tissue that is completely cleared (optically) provides enhanced contrast between cellular structures and may be what is necessary for a pathologist in order to determine a diagnosis with a given CB sample.

By using a custom-written algorithm for image registration and stitching along with OCA application (figure 4), future work will consist of z-stack imaging at high magnification across the *entire* CB (combining methods from figures 4 and 7). In this future case, it will be possible to visualize entire, intact, optically cleared CBs 1-2 cm long, 500-600 µm in diameter with high lateral and axial spatial resolution. In considering a single focus plane within the z-stack (figure 7a), a traditional 40x objective coupled with our CCD camera yielded a field-of-view consisting of ~2 megapixels. When combined with image registration and stitching, we may extend this to a maximum of 350 megapixels for a single focus plane in a given stack. Preliminary demonstration of these experiments has been presented elsewhere by our group25.

Finally, PBS, 50/50 PBS/glycerol and FocusClear® OCAs demonstrated a harmonic increase in the total transmitted light intensity in comparison to the same sample immersed in formalin solution volumes. Currently, most OCAs consist of a glycerol-, or DMSO-base (FocusClear®)30-31. Glycerol in particular is known to demembranate cells (i.e., "skinned muscle fibers"), hence it removes a main barrier for photon transmission. Consequently, initial results with 50/50 PBS/glycerol proved consistent with published literature30 and provide evidence for its use as an alternative, inexpensive OCA that is readily available. CBs treated with FocusClear® demonstrated similar increases in light transmission to tissue slices and may be explained by the thick capillary glass wall (despite refractive index matching) and the air gap between the enclosed CB and the top of the capillary tube (figure 2c).

This paper has presented the first steps at imaging entire CBs from pancreatic tissue using the same brightfield optical imaging technique used by pathologists. By adding specific staining for intracellular features like what was shown for islet cells, 3D images can be generated from intact CB with clinical diagnostic potential.

### 5. ACKNOWLEDGEMENTS

We would like to acknowledge Dr. Jennifer LaPointe from the Pathology Department, University of Washington Medical Center for helpful discussions on biopsy processing and procuring pancreas tissue slices. This work was supported by NSF-EAGER grant CBET-1212540.

### 6. REFERENCES


