Analyzing spatial correlations in tissue using angle-resolved low coherence interferometry measurements guided by co-located optical coherence tomography

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Abstract: Angle-resolved low coherence interferometry (a/LCI) is an optical technique used to measure nuclear morphology in situ. However, a/LCI is not an imaging modality and can produce ambiguous results when the measurements are not properly oriented to the tissue architecture. Here we present a 2D a/LCI system which incorporates optical coherence tomography imaging to guide the measurements. System design and characterization are presented, along with example cases which demonstrate the utility of the combined measurements. In addition, future development and applications of this dual modality approach are discussed.

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1. Introduction

Angle-resolved low coherence interferometry (a/LCI) is an optical technique used to study nuclear morphology in biological samples [1]. The a/LCI system operates by collecting elastically scattered light from biological samples for various scattering angles and utilizes interferometry to depth gate the measurements. The angular scattering distribution arising from selected depths within the sample can then be analyzed to determine quantitative depth-resolved nuclear morphology measurements [2]. The nuclear morphology data, specifically enlargement of the nuclei in the basal layer of the epithelium, have been shown to be a highly useful biomarker for the early detection of dysplasia, a precancerous tissue state. Previous studies have utilized a/LCI for evaluating epithelial dysplasia, including esophageal [3], colon [4], and cervical cancer [5].
Recently, the technique has been further developed into a two dimensional technique, 2D a/LCI, which has the capability to detect the entire scattering field in two transverse scattering planes for various depths within the sample to provide a more detailed description of the scattering structure [6]. A Fourier relationship exists between the angular scattering distribution and the spatial correlation function of a sample, enabling analysis of sample structures [7]. This approach can be extended to 2D Fourier analysis to study scatterer orientation and long range correlations within a scattering sample. As a 2D technique, a/LCI has been previously applied to characterize the structural information of a soft lithography phantom containing hexagonal-packed circular features [8]. In this paper, we now seek to apply this approach for characterizing tissue. However, as we will show, better visual guidance is needed to orient the 2D a/LCI measurements.

Although a/LCI shares a common depth gating scheme with optical coherence tomography (OCT), the approaches have not been previously combined. OCT is well established as a biomedical optical imaging technique that utilizes reflected and scattered light from a sample to produce depth resolved images up to 1-2 millimeters below the tissue surface with micron depth resolution and a few microns of lateral resolution [9]. OCT has been combined with a variety of optical techniques in multimodal platforms for enhanced characterization of biological tissues [10–12]. Recent examples include work by Maher et al., who developed a co-localized confocal Raman spectroscopy OCT system to study the spatial distribution of topically applied drugs within tissue samples [10], and by Wang et al., who developed a combined two-photon luminescence and OCT imaging system for characterizing atherosclerotic plaques [11]. Liu et al. demonstrated a multimodal imaging system which combines photoacoustic microscopy and OCT to measure the metabolic rate of oxygen in small animals \textit{in vivo} [12].

In addition to a/LCI, other interferometric techniques have been used to study sub-cellular structures in tissue. For example, optical coherence microscopy provides high spatial resolution and has been previously used to study morphological changes in neoplastic tissue [13]. Interferometric synthetic aperture microscopy (ISAM) reconstructs the 3D distribution of scattered light from tissue with isotropic resolution equivalent to the resolution at the focal plane [14]. In addition, micro-optical coherence tomography can greatly enhance the resolution of OCT as a tool for studying cellular and subcellular structures [15]. The Fourier transform light scattering (FTLS) technique obtains scattering information from measurements of the optical phase and amplitude for a thin sample [16, 17]. Despite the high spatial resolution of these techniques, a/LCI offers the distinct advantage of subwavelength measurements of scatterer size with high depth of field while also avoiding the need for an expert observer to interpret image features.

Here we present a multimodal system that combines 2D a/LCI and OCT to provide a unique analysis of sample structure that cannot be obtained using a single modality. The sequential scan of these two modalities in the same co-registered field of view (FOV) ensures proper sample orientation and permits correlation of the 2D a/LCI depth-resolved scattering measurements to specific tissue layers identified in OCT scans. The a/LCI measurements provide nuclear morphology information that is not typically accessible by OCT imaging alone. The analysis of scattering information is further extended to examine tissue correlations, up to a few hundred microns in length scale that are not detectable with the focused beam of OCT. We present two examples of combined a/LCI measurements and OCT imaging on \textit{ex vivo} tissue samples to demonstrate the utility of correlation measurements using the combined imaging system.

2. Instrumentation

The Fourier 2D a/LCI system has been described previously [6, 8] and is shown in Fig. 1(a). Briefly, light from a Ti:Sapphire laser (Coherent, Mira-900F, $\lambda = 830$ nm) is propagated into a spool of polarization maintaining fiber (50 m, Corning) for spectral broadening via self-
phase modulation. The spectrally broadened light has a bandwidth of 45 nm (FWHM) and is p-polarized by a linear film polarizer before it is split into the sample and reference arms of a Mach-Zehnder interferometer. Light in the sample arm is collimated and delivered to the sample (400 µm beam diameter, 20 mW optical power) at an incident angle of approximately 15°.

Light scattered by the sample is propagated to the entrance slit of the imaging spectrometer (Princeton instruments, SP-2150) which is located at a conjugate Fourier plane to the sample. The sample field is interfered with the reference field at BS3 before detection. For parallel acquisition of light in one scattering plane, a 640 by 480 pixel CCD array (AVT, Pike F-032) is used as a sensor for the imaging spectrometer and numerical dispersion compensation is performed to reduce unbalanced dispersion between reference path and sample path [6]. In contrast to OCT, where a spatial scan is implemented, the galvanometer in the a/LCI scheme sweeps across the Fourier plane of the scattered field, allowing line scan detection of the angle-resolved scattered field over a range of solid angles. This detection scheme achieves a total angular range of 0.59 rad (34°) along the scanning direction (θ₁, Fig. 1(c)) and 0.37 rad (21°) along the parallel detection direction (θ₂, Fig. 1(c)) as marked by the yellow box in Fig. 1(c). The system achieves a full width at half maximum (FWHM) angular resolution of 0.137° and scanning was performed with an angular step size of 0.057°. Comparison of the scanning ranges for 1D and 2D a/LCI scattering measurements is shown in Fig. 1(c). The 2D a/LCI scan acquires two orders of magnitude more scattering measurements.
over two scattering directions compared to an individual 1D a/LCI scan. Thus, the 2D a/LCI measurement provides more detailed information of a sample’s scattering structure, including anisotropy of scatterers [18]. The system acquires one scattering plane in 4 ms (1 ms integration, 3 ms read out), and acquires an entire 2D scattering field (600 angular scans) in approximately 2 seconds.

The unique aspect of this system is the integration of an OCT scanner for direct comparisons of a/LCI measurements and OCT images of the same tissue sample. The system integrates a commercial research OCT system (Wasatch Photonics, Spark, \( \lambda = 840 \text{ nm}, \Delta \lambda = 155 \text{ nm} \)) which has 1.9 mm of imaging depth with measured axial and lateral resolution of 2.6 \( \mu \text{m} \) and 12.3 \( \mu \text{m} \), respectively. The OCT beam is relayed through a 4f system to the sample plane, and a flip mirror is inserted within the relay for sequential imaging between the two imaging modalities, co-registered to the same FOV as shown in Fig. 1(b) and 1(d). In order to overlap the a/LCI beam with OCT FOV, the 400 \( \mu \text{m} \) a/LCI beam is passed through a 1 mm diameter pinhole at the sample plane, and then the pinhole is centered in the 5 x 5 mm\(^2\) OCT FOV at the identical sample plane. A 3D OCT scan of the pinhole is shown at the sample plane in Fig. 1(b). Short focal length lenses (35 mm) are chosen for the 4f system to ensure a high enough NA to achieve the desired angular collection range for a/LCI measurements.

In a previous study, it was shown that the Fourier transformation of the angular scattering distribution yields the two-point correlation function of the optical field in the Born approximation [7]. Therefore, through the Fourier relationship, the achieved spatial resolution of the correlation function is determined by the measured angular range of the scattered field. Similarly, the greatest detectable correlation length is determined by the angular sampling resolution of the scattered field. With the angular resolution and range along the two directions in the a/LCI measurement given above, the theoretical resolution and range in the correlation domain was determined to be 1.7 \( \mu \text{m} \) and 420 \( \mu \text{m} \) along the scanning direction and 2.7 \( \mu \text{m} \) and 440 \( \mu \text{m} \) along the parallel detection (non-scanning) direction. However, in practice, the angular resolution along the vertical (scanning) direction is degraded due to the continuous motion of the galvanometer during acquisition and the higher degree of spherical aberration due to the off axis geometry. Experimentally, the implemented system achieves a FWHM spatial resolution for measuring correlations of 3 \( \mu \text{m} \) and 6 \( \mu \text{m} \) along the horizontal (parallel, non-scanning) and vertical (galvanometer scanning) directions, respectively. The maximum correlation distance is effectively limited by the 400 \( \mu \text{m} \) beam diameter in both directions.

In order to calibrate the 2D a/LCI system and assess the validity of the autocorrelation method, a scattering phantom with a high degree of radial and self-symmetry was constructed via soft lithography using polydimethylsiloxane (PDMS) as shown in Fig. 2(a) [8]. The hexagonal packed circular scattering features have a 12 \( \mu \text{m} \) diameter with a 24 \( \mu \text{m} \) center to center separation between adjacent features. The en face OCT image of the sample (Fig. 2(b)) does not have high enough lateral resolution to provide size measurements of the scatterers. The measured scattering distribution by 2D a/LCI for the depth plane corresponding to the phantom features is shown in Fig. 2(c). The information in the scattering plane is specified by the vertical and horizontal polar angles (\( \theta_1 \), and \( \theta_2 \), respectively) which facilitates Fourier analysis. The range of \( \theta_2 \) is chosen to be symmetric about the origin, but \( \theta_1 \) is unidirectional in order to avoid the specular reflection from the sample. The vertical polar angle \( \theta_1 \), which is the scanning axis, has a higher angular range than the horizontal polar angle, but it has a broader impulse response due to the galvanometer motion, as discussed above. The correlation distribution from the phantom shown in Fig. 2(d) highlights the hexagonal packing of the structural elements. Note that the spatial distribution of the scatterers in the correlation plane reveals not only the spacing between the features, but also directional information of the scatterers. When the 2D correlation energy is integrated azimuthally at each radial distance, the distance between peaks in the radial correlation energy has been calibrated to match the spacing of elements within the phantom (Fig. 2(e)).
Fig. 2. Images of calibration phantom constructed via soft lithography in PDMS. The scatterers have a nominal 12 μm diameter with a 24 μm center to center spacing. (a) Bright field microscopy image, (b) OCT en face image (x-y) with a cross section (x-z) in the inset, (scale bar = 100 μm, inset scale bar = 30 μm), (c) 2D a/LCI measurement (angular plane), (d) Fourier transform of 2D a/LCI measurement (correlation plane), and (e) azimuthal summation of (d).

One additional modification is included in this combined system. A custom designed objective lens is used for imaging intact mouse eyes and inserted in front of lens L2 as shown in the inset of Fig. 1(b). This objective and the mouse eye lens form a 4f configuration that images the retina onto the sample plane of the combined system. The objective serves to correct the aberrations from the mouse eye lens. The eyeball is inserted in a sample chamber that matches its curvature to maintain alignment of the eyeball relative to the objective lens during translation to different regions of interest (ROIs). The sample chamber is then placed on a translational stage to allow fine adjustment along the x, y, and z directions. The front surface of the eyeball is placed in contact with the objective lens as shown by the dotted lines in the inset of Fig. 1(b). Prior to acquiring the 2D a/LCI measurements, OCT imaging is first utilized to align the optic nerve head (ONH) at the center of the FOV in the retinal image, where the a/LCI beam is aligned to the sample. The centered ONH (Fig. 3) functions as the reference point used to designate the locations of the ROIs imaged with the system.

Fig. 3. OCT imaging of retinas for (a) wild type, and (b) Rhodopsin knockout mice. The location of the ONH is indicated with the white arrow, and serves as the reference point to designate the locations of the ROIs imaged. The scale bar indicates 100 μm.

3. Sample preparation

Two types of ex vivo tissues have been examined with the 2D a/LCI instrument, epithelial tissues from a rat model of esophageal adenocarcinoma and intact mouse eyes, from either a wild type animal or a genetically created model of retinal degeneration. All the animal procedures for both the animal models were approved by the Duke Institutional Animal Care
& Use Committee (IACUC). In the rat model, an esophagogastroduodenal anastomosis (EGDA) is performed to create chronic reflux of bile salts and stomach acid, mimicking reflux driven Barrett’s Esophagus in humans [3]. Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) at 5-6 weeks of age. Following a one week acclimation period to the facility, rats were randomized to experimental groups. Rats were fasted overnight for approximately 16 hours prior to surgery. Aseptic surgical techniques were strictly adhered to throughout the anastomosis procedure. Rats were continuously monitored throughout surgery and immediately post-surgery. Once the animal was deeply anesthetized a 1 inch upper midline incision was made inferior to the sternum to open the abdominal cavity, and the esophagus, stomach and duodenum were exposed via blunt dissection of fascia and retraction with sterile gauze. Two 1.5 cm long incisions were made; one at the gastroesophageal junction (not reaching the glandular stomach), and one on the anti-mesenteric border of the duodenum (approximately 1 cm from the pylorus). A side-to-side duodenoesophageal anastomosis was performed with accurate mucosal to mucosal opposition. After careful lavage, the peritoneal and muscle layers were closed with absorbable suture and Vetbond Tissue Adhesive (3M) was used to close the dermal layer, followed with 4 to 5 skin staples. After the surgery was completed, the rat was injected with 5 mL of sterile saline subcutaneously in the skin over the back and anesthesia was turned off. Buprenorphine was utilized to provide pain relief for 72 hours, after which the rats were mobile and fully active.

Imaging of the mouse retina was performed using intact eyeballs from wild type or rhodopsin knockout (RhoKO) mice of different ages (4, 8, and 12 weeks old). Rhodopsin knockout causes a progressive retinal degeneration, which makes this mouse a useful model of human disease conditions, such as retinitis pigmentosa [19]. The eyes were dilated with a solution containing 1% cyclopentolate and 2.5% phenylephrine for 6 minutes. Mice were then euthanized with CO₂ asphyxiation, followed by decapitation. The superior part of each eye was marked, and the eyes were removed from the skull and stored in cold mouse Ringer’s solution consisting of 130 mM sodium chloride, 2.5 mM potassium chloride, 1 mM magnesium chloride, 28 mM glucose, 10 mM HEPES and 2 mM calcium chloride (pH 7.4). The eyes were cleaned of connective tissue and muscle, the optic nerve was severed, and the eyes were placed into a greased divot in the custom-designed tissue chamber described above. The eyes were positioned so that the superior-inferior quadrants were oriented top-bottom, and the ONH was oriented in the center of the dilated pupil. The eye was immersed in the Ringer’s solution during imaging.

4. Results

The co-registered 2D a/LCI and OCT modalities in the combined system provide unique information to help interpret the structure of a sample based on light scattering data. Since the a/LCI beam has a 400 μm beam diameter, it is crucial for the sample to be oriented such that layered structures are even across the FOV to effectively retrieve and analyze the depth resolved scattering information. Therefore, OCT images were used to ensure proper sample orientation and to register the 2D a/LCI depth measurements to the histological layers. Two examples of combined a/LCI measurements and OCT imaging are presented to demonstrate the utility of this multimodal imaging system.

4.1 Rat esophagus

Figure 4 shows OCT images and depth resolved a/LCI light scattering data collected from an ex vivo tissue sample from the rat model of esophageal carcinogenesis. To contrast the quality of the a/LCI data with sample orientation, two examples are shown, one where the histological layers are flat across the scan and one where tissue folds are present. When the sample is oriented so that the tissues layers are flat, as shown by the OCT image in Fig. 4(a), the corresponding a/LCI scan (Fig. 4(b)) at the same ROI shows sharp transitions between
each tissue layer. However, when the sample has undesired tissue folds, as shown in the OCT image in Fig. 4(d), the resulting a/LCI scan (Fig. 4(e)) shows blurred tissue layers, making it difficult to register and analyze the a/LCI scan. The depth measurements in the OCT and a/LCI scans are given in units of optical path length (OPL) instead of physical distance where OPL is defined as the refractive index times the physical distance. While OCT can resolve histological layers due to its high axial resolution, most implementations lack the transverse resolution to measure sub-cellular features such as nuclear diameter. Further the limited spatial dimension of the focused beam in OCT prevents measurements of long range spatial correlations. However, both subcellular features and long range correlations can be extracted from a/LCI measurements. For example, nuclear diameter and cell to cell spacing have been accurately measured by Pyhtila et al. [20] using 1D a/LCI measurements of micro patterned cell arrays.

The 2D a/LCI data can be used to gain more information about the tissue sample structure than OCT imaging alone by analyzing sample correlations and through model based analysis. In order to compare correlations across different depths, the correlation at each depth is normalized by the corresponding scattering intensity so that they are independent of the overall reflectivity. Figure 5(a) shows the total normalized correlation as a function of depth in the sample for the esophageal tissue, obtained by Fourier transforming the 2D a/LCI scan and then integrating across solid angle. As can be seen, certain regions, corresponding to different depths beneath the tissue surface, show increased correlation. The increase in tissue organization can be linked to the epithelial layers using the OCT image. For example, the correlation energy can be used to highlight the presence of cell nuclei. Figure 5(b) shows the correlation energy binned across radial lengths from the 10 to 13 μm range, the expected size of cell nuclei in this epithelial tissue. The tissue feature marked as ROI shows higher correlation energy at this specific length scale compared to the same depth in the overall correlation function shown in Fig. 5(a). However, examining the correlation energy for a similar range of correlation lengths but at larger spatial scales (19-22 μm, Fig. 5(c)) shows a lower correlation. While many of the peaks show consistent magnitudes in the correlation...
plots (Fig. 5(a)-5(c)), the peak at the 10-13 μm range at the selected depth is consistent with known cell nuclei size and localization at the basal layer of the epithelium in this model [1]. The basal layer is identified as laying slightly deeper than the highest intensity profile of the tissue, with the typical depth in previous studies of rat esophagus ranging from 50 to 100 μm. However, due to thickening of the keratin layer, localization of the basal layer as deep as 200 μm has been observed [21]. The selected depth in this study is located at approximately 160 μm in optical path length from the tissue surface while the highest intensity peak is located at 140 μm in optical path length from the surface. Assuming the index of refraction of the mice epithelial tissue to be 1.37, consistent with other epithelial tissues [22], the physical depth is approximately 115 μm for the selected ROI.

Further details of the scattering structure can also be obtained through analysis of the correlation data. Figures 6(a) and 6(b) show the angular scattering intensity and 2D correlations for the same depth indicated by ROI in Fig. 5(a)-5(c). The azimuthally integrated 2D correlation energy (Fig. 6(c)) at the ROI reveals 2 peaks at 11.6 μm and 16.7 μm length scales but otherwise drops off monotonically. As shown previously, these correlation lengths are roughly consistent with the cell nucleus size and cell to cell spacing [7]. However, simple Fourier based analysis using the correlation function analysis provides limited resolution (1.7 μm and 2.7 μm in θ1, and θ2 directions in theory, but 6 μm and 3 μm measured) which may not be sufficient to discriminate the subtle nuclear size changes that reveal early cancer [3].

To reveal the structure of the cell nuclei more precisely, the 2D angular scattering measurements can be analyzed using a theoretical model such as Mie theory [2] or T-matrix based analysis [23]. While T-matrix based analysis can give size, shape, and orientation of scatterers by treating scatterers as spheroids instead of spheres as in Mie theory, it requires significant computation time. Instead, analysis can be simplified by extracting a 1D scattering profile from the 2D angular scattering measurement for comparison to Mie theory simulations as shown in Fig. 6(d). As described previously, the diameter of the scatterers is determined by minimizing the difference between experimental data and simulated solutions across a range
of scatterer sizes and relative refractive index of the sphere to the surrounding. From the 2D a/LCI angular map, a single 1D scattering profile ranging from $\theta_1 = 0^\circ$ to $30^\circ$ and $\theta_2 = 0^\circ$ was selected for the Mie fitting. For the 1D scattering profile extracted from the ROI, the scatterer diameter is determined to be 9.7 $\mu$m, with index of the refraction of nuclei to be 1.43, and the surroundings to be 1.37, in good agreement with a previous measurement of normal rat esophageal epithelium [1]. When the same layer from the folded tissue was fitted with Mie theory, the goodness of the fit was much worse than that calculated from the properly oriented tissue sample and no scatterer measurements were obtained.

In addition to determining the size of scatterers by analyzing short range correlations (less than 20 $\mu$m), the monotonically decreasing correlation trend at longer correlation lengths can also provide useful information for tissue characterization. The long range correlations are analyzed by fitting the monotonically decreasing component using a power law relationship: $I = A c^{-\alpha}$ where $I$ is the intensity, $A$ is the scaling variable and $\alpha$ is the power law exponent. The data over the 20-90 $\mu$m range of correlation lengths in Fig. 6(c) are fit by the two variables in the power-law relationship. This approach has been used previously to characterize rat esophageal tissues using a fractal dimension formalism [1, 20]. While fractal dimension and power law exponent can be simply related, we choose to present these results as a simple fitted parameter, the power law exponent.

![Fig. 7. (a) Normalized a/LCI intensity for the 0.225% 260 nm polystyrene beads solution, the esophageal tissue, and white paper, and (b) radial correlation energy vs correlation length by azimuthal integration of 2D a/LCI correlation plane at the same depth for each sample in log-log scale. The blue box indicates the region where data were analyzed using the power-law fitting. (c) Amplitude of fitted power law exponent across entire imaging depth for each sample. Depths in (a) and (c) are in optical path length.](image)

To illustrate the power-law fitting technique, two technical samples were imaged and the long range correlations were analyzed. Figure 7(a) shows normalized a/LCI intensity as a function of depth for three samples: a solution containing 0.225% 260 nm diameter polystyrene beads (Duke Scientific Corporation), the rat esophageal tissue, and white card stock (thick paper). The a/LCI measurements for these three samples were aligned so the air/sample interface appears at a depth of 0.4 mm. The radial correlation energy at each depth for each sample was calculated by Fourier transforming and azimuthally integrating the 2D correlation field of the a/LCI measurements as described above. For each sample, depth ranges of 30 $\mu$m intervals were binned for correlation analysis. The radial correlation energy for all three samples is shown on a log-log scale in Fig. 7(b), alongside an equivalent measurement where no sample was present as an indication of system noise. These correlation energy measurements are obtained from the same depth as the ROI indicated in Fig. 5(a)-5(c). Note that a more rapid fall off occurs in the correlation measured from white card stock and esophageal tissue at long correlation lengths, as compared to that from the polystyrene beads solution (measured $\mu_s = 0.51$ mm$^{-1}$). The power law exponent was calculated using a least square fitting technique for the data across the 20 to 90 $\mu$m correlation length scales as indicated by the blue box in Fig. 7(b). The magnitude of the fitted power law exponent ($\alpha$) for each sample over depth is plotted in Fig. 7(c). The noise plot shows uniform correlation across all depths as expected, while the polystyrene bead solution has a constant...
power law exponent across all depths except a sudden increase at the air/water interface. The magnitude of the power law exponents measured for the white paper and esophageal tissue also show a decreasing trend over depth, but much higher values than that of beads. This trend indicates that the correlation falls off more quickly for these two samples compared to the correlation of the bead samples. Finally, the fitted power law exponent for the white card stock decreases monotonically over the imaging depth, in contrast to the esophageal tissue which shows overlying structure at certain depths.

4.2 Mouse retina

The 2D a/LCI measurements of mouse retina were collected at 8 locations ranging radially from 500 μm to 1.5 mm from the ONH. Each scattering measurement was collected with an acquisition time of approximately 750 ms. For each 2D a/LCI measurement, an OCT image of the same ROI was collected. Representative data from a healthy mouse retina, consisting of an OCT image and an a/LCI scan of scattered light intensity measured as a function of depth and polar angle, are shown in the Fig. 8(a), and 8(b). Note that the a/LCI scan is a function of scattering angle but extends over 400 μm in the lateral direction, a much smaller region than that shown in the OCT image (Fig. 8(a)). The OCT scans of both wild type mice and RhoKO mice are segmented manually by following the OCT segmentation methodology for murine retinal images given by Srinivasan et al. [24] to enable correlation of the light scattering data with specific histological layers. Angle-resolved data are analyzed at each depth to determine spatial correlations in the transverse plane. For this particular experiment, the angular scattering information was analyzed for the nerve fiber layer (NFL), outer plexiform layer (OPL), and the layer containing both outer segment photoreceptors and retinal pigmented epithelium (OS/RPE) as the three layers have strong scattered intensity and histological changes at the OPL layer are expected in this model due to rapid photoreceptor degeneration. A previous study by Wang et al. showed the angular dependence of scattering at the NFL layer in human subjects by separating low and high angle scattering via path-length-multiplexed scattering-angle-diverse OCT (PMOCT) [25] and thus this layer may be of interest for diagnosing early retinal disease.

![Fig. 8. (a) OCT scan of wild type mouse retina (scale bar = 100μm). (b) Scattering intensity collected via 2D a/LCI as function of depth vs polar angle θ. Arrows indicate corresponding layers.](image)

To illustrate typical data, the 2D a/LCI scattering distribution and the corresponding correlation function for the OS/RPE layer of a healthy mouse retina are shown in Fig. 9(a) and 9(b), respectively. Note that the collected angular range in the intact mouse eye was smaller compared to that of the calibration phantom in Fig. 2(c) due to the physical aperture of the mouse pupil.

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Fig. 9. (a) Scattering plane from the same sample at the depth corresponding to outer segment retinal pigmented epithelium layer (OS/RPE), and (b) correlation plane of the OS/RPE.

The azimuthally integrated 2D correlation energy of the scattering from the OS/RPE layer shown in Fig. 9(b) above is shown in Fig. 10(a). In contrast to the radial correlation energy of the esophageal tissue, which had distinct peaks for correlation length scales less than 20 μm and then drops off monotonically afterward, that of the retinal tissue showed a monotonic decrease in correlation throughout. To compare healthy and diseased tissues, the correlation data were again integrated azimuthally and presented as normalized radial correlation energy versus correlation length in log-log scale forNFL, OPL, and OS/RPE layers in Fig. 10(b). The a/LCI measurements in the three layers were binned in 30 μm depth intervals. The typical depth selection in optical path length for the NFL layer was 50-70 μm from the tissue surface for both wild type mice and RhoKO mice. However, the typical depths selected for the OPL layer varied across the model. They were 220-260 μm for the wild type, 175-205 μm for the 4 weeks old RhoKO, 155-195 μm for the 8 weeks old RhoKO and 120-160 μm for the 12 weeks old RhoKO relative to the tissue surface. Similarly, the typical depths selected for the OS/RPE layer also varied across the model. They were 310-370 μm for the wild type, 260-300 μm for 4 weeks old RhoKO, 240-280 μm for 8 weeks old RhoKO, and 190-250 μm for 12 weeks RhoKO. Changes in the depth selection for different stages of the model are to be expected due to the progressive degeneration of photoreceptors in RhoKO model. The decay of the correlation energy with length is similar for the OS/RPE and NFL layers but is different for the OPL layer. To quantitatively compare the correlation energy in each layer for the wild type and RhoKO mice, the power law exponent was determined across the 20-90 μm range at each depth as indicated by the blue box. The power law exponent was evaluated for wild type mice and RhoKO mice ranging from four to twelve weeks of age, which is the time window during which retinal degeneration progresses in these animals. (Fig. 10(c)). The analysis shows that power law exponent exhibits significant differences for some layers between normal and RhoKO animals. For the OS/RPE layer, the RhoKO had a p-value less than 5.8E-10 with two sample t-test for all time points when compared to the wild type. For the OPL layer, a significant difference (p-value of 3.1E-5) was observed only for 12 week old RhoKO mice, the age at which retinal degeneration is mostly completed. No statistically significant changes are seen for NFL, which is consistent with this part of the retina being essentially spared from degeneration in this model. The scatterer’s size fit across different layers and types of animals with Mie theory was unsuccessful due to the limited angular range collected through the mouse pupil and the imperfection of the optical properties in the mouse lens. The chi-squared value of the best fit was determined non-unique, often yielding different size of scatters with non-converging solution, indicating that no nuclear sizes could be determined for these tissues.
5. Discussion

We presented a multimodal system that combines 2D a/LCI and OCT in a co-registered FOV to provide a unique characterization of sample structure that cannot be obtained using a single modality. The OCT system serves as image guidance for the 2D a/LCI depth measurements and ensures proper sample orientation and co-registration of light scattering measurements to histological layers, providing a more effective analysis of the depth resolved scattering information. The OCT guided, 2D a/LCI scattering field reveals nuclear and cellular morphology information and long range correlation measurements that are typically inaccessible by OCT imaging alone. The application of the combined system to \textit{ex vivo} tissue samples shows the potential utility of the system for discriminating disease states.

When imaging rat esophageal tissue, the OCT image was used to ensure flat tissue orientation with respect to the illumination beam allowing each layer in the a/LCI image to be co-registered with the histological layers observed in the OCT image. By analyzing the correlations across varying spatial length scales at each depth, structural features of the sample are revealed that are not seen in conventional OCT images. Figure 5(a)-5(c) show that certain tissue layers have increased correlations at specific length scales. The azimuthally averaged radial correlation function in Fig. 6(c) shows decreasing correlation energy with increasing length scale. Further, two distinct peaks are observed at correlation lengths of 11.6 μm and 16.7 μm, consistent with the cell nucleus size and cell to cell spacing. The Fourier based analysis is limited in precision due to the resolution of the correlation function (3 μm). However, Mie based analysis provides a more precise measurement of scatterer diameter. The diameter of the scatterers was determined to be 9.7 μm with a nuclear refractive index of 1.43 and a cytoplasm refractive index 1.37. Thus the correlation peak agrees with the Mie theory analysis within the resolution of the Fourier analysis. Both the Fourier based correlation analysis and the Mie fit results were in good agreement with previous studies with this animal model [1, 20]. In order to ensure the uniqueness of the Mie fitting, the chi-squared value of the best fit is compared to the chi-squared value of the fit obtained with the null solution. Then the chi-squared value of the best fit is compared that of the next best fit. If the best fit value is not smaller than the second best fit by more than 10%, the fit is determined to be a non-unique solution and discarded [2]. Both the properly oriented and folded samples passed the first test criterion in the comparison with the null solution, indicating that useful scattering information was obtained. However, the chi-squared fit for the folded sample failed to pass the second criterion and was thus treated as a non-unique solution. Thus, using OCT imaging for real-time a/LCI guidance, measurements can be targeted to ROIs which are more likely to yield convergent Mie fits.

The use of a power law function to fit the angular scattering data was introduced with the original a/LCI technique [7] and then used for analysis of rat esophageal tissue data [1]. The inverse power law behavior indicates a fractal dimension (FD) which is a measure of the self-
similar nature of the tissue [26]. The power law exponent, $\alpha$, can be related to the fractal dimension by $D = 3 - \alpha$ [7]. In a previous study of squamous cell carcinoma in the rat esophagus, the calculated FD ranged from 2.06 to 2.42 using a 2 to 20 $\mu$m correlation range compared to 2.44 to 2.72 in this study. This is in good agreement with Yi et al., who showed the measurement of fractal dimension in biological tissue to be between 2 and 3 by recovering the refractive index correlation function using inverse spectroscopic OCT (ISOCT) by modeling tissue as a continuous medium with fluctuation [27]. Instead of modeling the scattering in tissue as a collection of spheres within the medium as is done in Mie theory, light scattering in tissue can be modeled as a continuous random medium [28] or can be described using an inverse scattering model [29]. Similarly, the power law fitting method shown here does not treat the tissue scattering as discrete scatterers but rather analyzes the medium as continuous.

In this paper, the power law exponent was determined by the least squares fitting technique across length scales from 20 to 90 $\mu$m correlation lengths. The lower end of the correlation range was chosen as 20 $\mu$m to study tissue features greater than the size of cells, a length scale not accessible by the Mie fitting method. The higher end of the range was chosen as 90 $\mu$m by finding the largest correlation length which still allowed good fits to the power law. As shown, in the data, the correlation function exhibits a falloff at longer length scales. Unlike previous analyses, the high throughput nature of these measurements and guidance by OCT imaging allows for a broader study. Depth resolved correlation analysis was conducted for a solution of 260 nm polystyrene beads, white card stock (paper), and rat esophageal tissue. For the bead solution, the value of the fitted power law exponent stayed constant over depth, matching our expectations since no long range correlations are expected. The measurements of white card stock illustrate that depth resolved correlation measurements depend on how the light has propagated to reach that tissue depth. Although the card stock had uniform structural properties, the power law exponent was seen to monotonically decrease as a function of depth. The esophageal tissue similarly showed a decrease in correlation for increasing depth but with distinct changes at specific depths. The monotonic decrease in the power law exponent as a function of depth indicates a reduction of spatial coherence in the light returning from deeper depths within the sample. This is due to a combination of wave front distortion and defocus aberration. As the collimated a/LCI beam travels through the sample, the plane wave front is modified due to forward scattering, effectively reducing its spatial coherence. Light that is scattered deeper within the sample forms spherical waves that are centered far from the focal plane of the imaging system, resulting in defocus aberration when detected by the imaging spectrometer. Both of these effects cause degradation of spatial coherence, which contributes to a falloff of the correlation measurements at greater depths. We note that a/LCI is most often used to analyze the basal layer of the epithelium which is typically less than 300 $\mu$m beneath the tissue surface. Thus a/LCI instruments are designed with the focal plane located approximately 100-200 $\mu$m beneath the surface. The observed depth dependence of the correlation measurement may explain why such a variance in FD values for tissue features are seen in literature.

The combination of OCT and a/LCI allows for better localization of structural features when analyzing the mouse retina model. In this case, the OCT image served as a guide to locate identical ROIs for a number of different samples. The ONH was located using OCT to position the 2D a/LCI measurement, serving as a reference point to designate the locations of the imaged ROIs. The power law exponent measured for RhoKO mice at the OS/RPE layer was significantly higher than that of wild type mice, whereas a difference in the power law exponent at the OPL layer was observed only in 12 week old RhoKO animals. These results demonstrate that a/LCI is not only capable of extracting depth-dependent cellular scale information based on the angular scattering field but also has the potential for early detection of degenerative retinal disease using long range correlation analysis.
6. Conclusion

In this study, we have demonstrated the utility of a combined OCT and 2D a/LCI system in imaging ex vivo tissue samples. OCT imaging serves as alignment guidance for locating specific ROIs as well as proper sample orientation at the selected region. Using our multimodal system, we verified that the rejection of improperly oriented tissue sites can reduce a/LCI post processing time for the samples that are more prone to non-unique nuclear size fits, as demonstrated with comparison of the folded and flat esophageal tissue. In addition to nuclear size determination, we measured the fractal dimension within the esophageal and retinal tissue, which confirmed the self-similar nature within the tissue. Finally, the comparison of the normal versus diseased retinal tissue revealed significant differences in morphology, and quantitative biomarkers were determined that could be used in future studies.

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