Abstract: There is potential clinical significance in identifying cellular responses in the anterior chamber (AC) of the eye, which can indicate hyphema (an accumulation of red blood cells [RBCs]) or aberrant intraocular inflammation (an accumulation of white blood cells [WBCs]). In this work, we developed a spectroscopic OCT analysis method to differentiate between populations of RBCs and subtypes of WBCs, including granulocytes, lymphocytes and monocytes, both in vitro and in ACs of porcine eyes. We developed an algorithm to track single cells within OCT data sets, and extracted the backscatter reflectance spectrum of each single cell from the detected interferograms using the short-time Fourier transform (STFT). A look-up table of Mie back-scattering spectra was generated and used to correlate the backscatter spectral features of single cells to their characteristic sizes. The extracted size distributions based on the best Mie spectra fit were significantly different between each cell type. We also studied theoretical backscattering models of single RBCs to further validate our experimental results. The described work is a promising step towards clinically differentiating and quantifying AC blood cell types.

1. Introduction

There are many diseases associated with cellular response in the anterior chamber (AC) of the eye, the anatomical space between the cornea and the iris which is occupied by the aqueous humor. For example, hyphema is a collection of red blood cells (RBCs) inside the AC, which can occur after blunt trauma, after intraocular surgery, or spontaneously [1]. On the other hand, anterior uveitis, the most common form of intraocular inflammation, causes an accumulation of white blood cells (WBCs, also called leukocytes) in the ocular anterior chamber. Further, the subtype of WBC response may provide differentiating diagnostic information regarding the type of inflammation and hence the appropriate treatment. For instance, a predominant response of polymorphonuclear WBCs (also called granulocytes), mainly neutrophils, may suggest an endotoxin-induced or HLA-B27-associated acute uveitis [2,3], while a predominant response of mononuclear WBCs, mainly lymphocytes and monocytes, may suggest sarcoidosis [4], Vogt–Koyanagi–Harada [5] and melanin-protein-induced uveitis [6]. The primary current diagnostic tool to examine the condition of the aqueous humor and evaluate the severity of these diseases is slit-lamp microscopy. Although specialists can recognize the presence or absence of these responses, subjective qualitative assessment of these conditions makes it difficult to judge the longitudinal course of the response. In addition, subtypes of WBCs, such as granulocytes, lymphocytes and monocytes, cannot be differentiated using clinical ophthalmic microscopy. The true composition of cells in the aqueous humor can be obtained using AC paracentesis and flow cytometry [7].
However, AC paracentesis is an invasive procedure that requires a needle insertion and local anesthesia [8].

Optical coherence tomography (OCT) allows noninvasive, high-resolution cross-sectional imaging of the AC [9]. Several groups have investigated its potential in grading AC inflammation by locating and counting cells within anterior segment OCT (AS-OCT) images [10–14]. However, quantitative analysis of the true composition of cells using OCT remains a challenge. Healthy RBCs have a biconcave shape which is 7.5 to 8.7 μm in diameter and 1.7 to 2.2 μm in thickness [15], while WBCs are more globular with a mean diameter range from 6 to 20 μm [16]. Conventional, clinical AS-OCT systems have an axial resolution of ~5-10 μm in tissue, and a lateral resolution of tens of μms. Therefore, individual cells appear as indistinguishable hyper-reflective spots in conventional AS-OCT images [17]. Rose-Nussbaumer et al. have investigated differentiation of RBCs and subtypes of WBCs using the reflectance intensity in OCT images [17]. Although the average reflectance distribution of each cell type was shown to be different with statistical significance, cell reflectance in OCT images can be easily affected by many factors, such as illumination laser power, optical alignment, and cornea opacities. Ossowski et. al have developed a method to detect and differentiate moving RBCs and WBCs using phase-sensitive OCT in vitro; however, the method relies on using a custom designed microfluidic device with a highly scattering substrate, and is difficult to implement in vivo [18].

Utilizing a broadband source, spectroscopic OCT can provide additional backscattering spectral information that is sensitive to scatterer structure, such as scatterer size, shape and geometrical distribution [19,20]. Spectroscopic OCT has been previously applied to assess hemoglobin concentration [21], study retinal oximetry [22], and evaluate skin burn severity [23]. All these applications were performed in bulk tissues but not at the single cellular level. Xu et al. have previously demonstrated that spectroscopic optical coherence microscopy can be used to localize nuclei within individual cells [24]. To our knowledge, this is the first time that a clinically viable spectroscopic OCT technology was developed at the single cell level with sufficient depth of focus to cover the AC.

In this work, we developed a spectroscopic OCT analysis method to track and extract the backscattering spectrum of isolated single blood cells, and to correlate the spectrum of individual cells to their characteristic sizes. Based on the characteristic size distributions, we were able to differentiate between populations of RBCs and subtypes of WBCs, including granulocytes, lymphocytes and monocytes, both in vitro and within the AC of ex vivo porcine eyes. Additionally, we investigated theoretical backscattering models of single RBCs to further validate and explain our experiment results.

2. Methods

2.1 OCT system design

A custom swept-source OCT (SSOCT) system (Fig. 1(a)) was built for this study. The system employed a 100-kHz swept-wavelength laser (Axsun Technologies; Billerica, MA), centered at 1052 nm with a sweeping range of 108 nm. The interferometric signal was detected with a dual-balanced receiver (Thorlabs, Inc.; Newton, NJ) with 1GHz electronic bandwidth and digitized at 800 MS/s (AlazarTech Inc.; Pointe-Claire, QC, Canada). The sample arm included a collimator lens, a pair of galvanometer scanning mirrors (Thorlabs, Inc.; Newton, NJ) and a telecentric imaging lens with a working distance of 75 mm (Fig. 1(a)). The lateral resolution was measured to be 20 μm. The measured sensitivity and ~6 dB fall off of the OCT system was 103 dB and 4.39 mm with 2.2mW illumination power on the sample, respectively (Fig. 1(b)). The measured axial resolution was constant over an imaging range of 5.5 mm with a mean full width at half maximum of ~8.8 μm (in air) (Fig. 1(b)). The maximum imaging range of the system was 7.4mm. Custom GPU-based software enabled real-time acquisition, processing, and rendering of volumetric data at a 100-kHz A-line rate.
Fig. 1. (a) Schematic diagram of the custom swept-source OCT system. L, L1, L2: lenses, G: galvanometer scanner, M: mirror, BD: beam dump, UP: unused port, BR: balanced receiver (b) fall off sensitivity performance and axial resolution of the system across an imaging range of 5.5mm

2.2 Phantom and cell preparation

2.2.1 Ex vivo phantom preparation

To first demonstrate the feasibility of using spectroscopic OCT to distinguish cellular scale objects in the AC, approximately 0.1 mL 10 µm polystyrene microspheres with a particle density of 1.05 g/cm³ (Thermo Scientific; Waltham, MA) were suspended in 5mL water and injected into the ACs of freshly enucleated porcine eyes (N = 2).

2.2.2 In vitro cell preparation

Fresh human RBC (N = 3) and cryopreserved human WBC samples (ZenBio Inc.; Research Triangle Park, NC), including granulocytes (N = 4), lymphocytes (N = 3) and monocytes (N = 3), from healthy donors were obtained. Cryopreserved WBC samples were rapidly thawed in a 37 °C water bath prior to use and suspended in phosphate-buffered saline (PBS) (Sigma-Aldrich; St. Louis, MO) solutions. Cryoprotectant was removed by centrifuging cell suspension at 400x g for 10 mins. The cell samples were then re-suspended in PBS solutions containing 0.1% bovine serum albumin (Sigma-Aldrich; St. Louis, MO) to a concentration of ~150 cells/mm³, and transferred to the glass cuvettes for imaging.
2.2.3 Ex vivo cell preparation

In the final experiments to mimic cell characterization in an in vivo environment, freshly enucleated porcine eyes obtained from a local slaughterhouse and stored at 4°C were used, and human RBCs, granulocytes, lymphocytes and monocytes (obtained and prepared as in Section 2.2.2) were injected into the ACs of porcine eyes using a syringe for immediate imaging.

2.3 Data acquisition

Due to the weak backscattering signal from single blood cells imaged by our system, we required between 20 and 30 repeated and averaged measurements from the same cell to achieve the necessary SNR for the following spectroscopic analysis (Fig. 3(c)-(d)). Unlike cells moving following a circulating aqueous humor flow pattern due to the natural temperature gradient in the eye [25], the flow patterns of in vitro or ex vivo cells can be affected by many factors, such as gravity, motion introduced during sample resuspension, and temperature gradients in the medium. Therefore, we found that two different OCT scan protocols, repeated B-scans and volumetric scans, were necessary, depending on the velocity and direction of cell movements observed in each sample, with the goal of keeping each single cell within the field of view (FOV) in repeated scans and, at the same time, maximizing the FOV to capture more cells.

For the data reported in this study, we used either volumetric scans or repeated B-scans at the same location. For each sample, we first aligned the sample such that the system’s B-scan direction was aligned along the dominant direction of cell motion (i.e., vertical due to gravity). Then, 50 repeated B-scans were acquired with 500 A-scans/B-scan, providing a FOV of 2 mm in the B-scan direction. For samples in which more than 50% of localized cells (according to the procedure described in Section 2.4) remained within the FOV for at least 20 repeated B-scans, the repeated B-scan data was used for further analysis.

For samples in which fewer than 50% of localized cells remained within the FOV for 20 repeated B-scans, a volumetric imaging window was utilized, particularly for freshly resuspended cells with higher and less uniform velocity, which were more likely to move out of the imaging plane in the B-scan mode. Instead, for these samples, 50 repeated OCT volumes were acquired with scan dimensions of 96 A-scans/B-scan and 48 B-scans/volume, and a lateral FOV of approximately 0.4 mm x 0.2 mm. The effective volume rate with these scan parameters was 22 Hz.

Both volumetric and repeated B-scan modes achieved nearly isotropic sampling resolution with a lateral sampling resolution of approximately 4 µm, and an axial sampling resolution of 5.4 µm.

2.4 Single cell localization and tracking

In the repeated B-scan mode, individual cells within OCT image (Fig. 2(a)) were first localized by creating a binary image using intensity thresholding (Fig. 2(b)), then 8-connected component labeling was applied to detect connected regions in the binary image and assign labels to individual cells (Fig. 2(c)). Additionally, if the distance between two cell labels were within 4 pixels, these two labels were combined as the label of the same cell. To differentiate cells from noise and exclude cells which were out of the imaging plane, only connected-components larger than 6 pixels were considered as cells. We used the A-scan (Fig. 3(a)) at the center of every labelled cell (Fig. 2(d)) for further spectroscopic analysis.

For the volumetric OCT mode (Fig. 9(a)), we performed the same processing as just described on each B-scan of each volume. If the distance between any centers of labels in two adjacent B-scans was less than or equal to 4 pixels, these two labels were combined as the label of the same cell. Similarly, we used the A-scan at the center of every labelled cell in the volume for further spectroscopic analysis.
To identify cells within sequentially acquired volumes, we compared whether the location of a cell in one volume corresponded with cell locations within a small voxel region in both the preceding and subsequent volume. If so, co-localized cell locations in sequential volumes were categorized as a single cell. The dimensions of the voxel regions were determined based on the moving speed of cells in each sample. The tracking of each cell stopped when it left the acquired volume. For B-scan mode, every single cell was tracked with a similar approach to locate cells in a neighborhood region. The cell was labelled to be out of the FOV when the peak cell intensity fell below a defined threshold value. Only cells staying in the FOV for at least 20 repeated B-scans or volumes were used for the following spectroscopic analysis.

Fig. 2. (a) Representative B-scan of lymphocytes suspended in the cuvette; (b) Binary B-scan image after intensity thresholding; (c) Individual cell localization using 8-connected component labeling, with each cell assigned a different overlay color; (d) Same B-scan as (a) with the centers of cells labeled.

2.5 Spectroscopic analysis

The ability to perform SOCT in Fourier-domain OCT using the STFT was first demonstrated by Letigeb et al. [26]. The signal intensity $I(k_z, z)$ extracted from the STFT of the detected interferogram $I_o(k)$ after linear wavenumber interpolation and DC subtraction can be expressed as

$$I(k_z, z) = \left| \text{FT} \left[ I_o(k) W(k - k_o) \right] \right|,$$

where $W(k - k_o)$ is the window function centered at $k_o$ and FT is the Fourier Transform. In this study, a Hamming window $W(k - k_o) = 0.54 + 0.46 \cos(2\pi \frac{k - k_o}{\Delta k})$ with a spectral width $\Delta k$ of 23.7 cm$^{-1}$ was chosen, corresponding to an axial resolution of ~340 µm for each window. To perform the STFT, the window $W(k - k_o)$ was shifted across the acquired interferogram $I_o(k)$ a total of 100 times with a step size of 9.2 cm$^{-1}$. Unlike for some previous spectroscopic OCT applications, the inherent tradeoff in spectral and spatial resolution of the STFT was not a significant drawback here, since the sample of suspended
cells was sufficiently sparse that the likelihood of having more than one cell within each depth window was low.

Assuming an optical path length difference between a point scatterer and reference arm is \( z \), the detected interferogram \( I_d(k) \) is proportional to \( S(k)\sqrt{R_i(k)} \cos(2kz) \), where \( S(k) \) is the laser power spectrum and \( R_i(k) \) is the backscattering power spectrum of the scatterer [27]. Thus, the signal intensity \( I(k_o,z) \) extracted from a window centered at \( k_o \) is

\[
I(k_o,z) \propto \left| FT\left( S(k)\sqrt{R_i(k)} \cos(2kz)W(k-k_o) \right) \right|.
\]

Assuming the backscattering power at the central wavenumber of the window, \( R_i(k_o) \), is the averaged backscattering power \( R_i(k) \) across that window, \( [k_o-\Delta k/2,k_o+\Delta k/2] \), and ignoring the complex conjugate,

\[
I(k_o,z>0) \propto \sqrt{R_i(k_o)} \left| FT\left( S(k)W(k-k_o) \right) \right| \delta(z-z_i)
\]

\[
= \sqrt{R_i(k_o)} \left| PSF(k_o,z) \right| \delta(z-z_i)
\]

\[
= \sqrt{R_i(k_o)} \left| PSF(k_o,z-z_i) \right|.
\]

Here \( PSF(k_o,z) = FT(S(k)W(k-k_o)) \) is the coherence function or axial point-spread function (PSF) of the “windowed” source spectrum. Experimentally, we obtained \( PSF(k_o,0) \) by imaging a mirror with a uniform backscattering profile \( R_i(k_o) \), which we used to calculate the normalized backscattering signal of the scatterer at \( z_i \), \( I_{norm}(k_o,z_i) \), as

\[
I_{norm}(k_o,z_i) = \frac{I(k_o,z_i)}{PSF(k_o,0)} \propto \sqrt{R_i(k_o)}.
\]

The backscattering spectrum of the cell, \( R_i(k_o) \), proportional to \( I_{norm}^2(k_o,z_i) \), was then calculated. The background spectrum of each cell was extracted from the adjacent 80 A-scans without cells at the same depth for noise subtraction. The spectrum after noise subtraction was then converted from wavenumber to wavelength and normalized to its mean (Fig. 3(c)). By tracking individual cells in repeated B-scans or volumes, the averaged spectrum of each cell with much higher SNR was obtained (Fig. 3(d)).
2.6 Mie theory library and fit

To estimate the backscattering spectra of polystyrene microspheres, we applied Mie theory to calculate the theoretical backscatter spectrum as a function of size for microspheres of a range of diameters. Although Mie theory assumes plane wave illumination, Xu et al. has demonstrated that, for an OCT system with a weakly-focused beam, the shape of the backscattering spectrum of a cell is not significantly affected by the NA of the sample beam, the axial location of the cell within the depth of focus, or the lateral location of the cell centered at within the beam width [28]. Thus, Mie theory is a reasonable model for this study.

A look-up table of Mie spectra was generated using MiePlot [29] with the following parameters: refractive index of polystyrene \( n_{\text{part}} = 1.572 \), refractive index of aqueous humor \( n_{\text{med}} = 1.336 \) [30], and the particle diameter \( d = 9-11 \) µm, in increments of 0.01 µm. We fit the normalized backscattering spectrum of each microsphere with the Mie spectra library we generated, and the best fit was determined by minimization of the mean squared error (MSE) between the Mie spectrum and our measured backscattering spectrum. The characteristic size of each microsphere was then directly extracted from the corresponding size of the best Mie spectra fit.

We also used Mie theory to correlate the backscatter spectra of single cells to their characteristic sizes. Previous studies have shown that, for WBCs, light scattering from the cell in the backward direction is mostly due to scattering from the nucleus [31], and the characteristic size extracted from fitting the oscillatory components in the spectrum is highly correlated to the size of the nucleus [32]. A look-up table of Mie spectra for multiple cell nuclei sizes was also generated using MiePlot [29] for comparison against our experimental spectroscopic data. We used the following parameters based on previous studies of refractive index profiles of WBCs using refractive index tomography [33,34]: the particle diameter \( d = 1-12 \) µm in increments of 0.05 µm; particle/nucleus refractive index \( n_{\text{part}} = 1.39-1.41 \), in
increments of 0.005; medium/cytoplasm refractive index $n_{med} = 1.35-1.37$, in increments of 0.005; and wavelength $\lambda = 998-1103$ nm.

2.7 Red blood cell backscattering spectrum modeling

Because RBCs lack cell nuclei and are better modeled as biconcave discs rather than spheres, Mie theory may not accurately model RBC cell size. Moreover, backscattering spectra of a single RBC will be dependent on the orientation of the cell in relation to the incident beam. Prior studies have investigated the backscattering spectra of a single RBC using first-order Born approximations [35,36], T-matrix [37], and finite difference time domain analysis [38]. Here, we applied a body of revolution spectral integral method (BoR-SIM) [39] to simulate the theoretical spectrum of an RBC model at different orientations. For this analysis, we used an expression for the shape of an RBC described by Fung, et al. [40] and Yurkin, et al. [41]:

$$T(x) = 0.65d\sqrt{1-x^2}\left(0.1583+1.5262x^2-0.8579x^4\right).$$  \hspace{1cm} (5)

Here, $T$ is the thickness of the RBC along the axis of symmetry, $x$ is a relative radial cylindrical coordinate [$x = 2\rho / d(-1 \leq x \leq 1)$], $\rho$ is a radial cylindrical coordinate, and $d$ is the diameter of the RBC. The diameter $d$ we used in our simulation was 7.8 $\mu$m [42]. The cross section and 3D shape of our idealized RBC model is shown in Fig. 4. We assumed the refractive index of RBC to be 1.39. We ignored the effect of absorption coefficient and refractive index differences between oxygenated and deoxygenated RBCs in our simulation, as for a single RBC, these spectral signatures due to absorption are significantly weaker than those oscillatory spectral features due to its geometry and orientation [35].

Assuming a plane wave incident field, we varied the angle of the incident field relative to the RBC to model the RBC at different orientations and the corresponding resulting backscattering spectra. Because the RBC model is radially symmetric along z-axis, varying the angle of incident field, $\alpha$, along one cross section plane, xz, was enough to cover all the possible incident field conditions (Fig. 4(b)). Additionally, we modeled the RBC backscattering cross-section spectrum at two orthogonal linear polarizations, horizontal polarization $\theta$ and vertical polarization $\Phi$, with the incident angles of field, $\alpha$, with respect to the RBC varying from 0° to 90° and a step size of 2.5°. Using a Mie theory fit, we compared the simulation results to the experimental results. While Mie theory cannot accurately model RBC cell size, as we also show later in Section 3.2 and 3.4, both the simulated and experimental results showed that the extracted characteristic size distribution difference was sufficient to differentiate populations of RBCs from all subtypes of WBCs.

Fig. 4. The (a) cross section and (b) 3D rendered shape of the RBC model. In (b), illustrative angles are shown of the incident field $\alpha$ (blue arrows), at two orthogonal linear polarizations, horizontal $\theta$ (green arrow) and vertical $\Phi$ (yellow arrow).
3. Results

3.1 Phantom results

A representative OCT B-scan image of 10 µm polystyrene microspheres injected into the AC of a freshly enucleated porcine eye is shown in Fig. 5(a). Representative spectroscopic data of a single microsphere and its best Mie spectral fit are shown in Fig. 5(b). A histogram of microsphere sizes (N = 121), extracted from the corresponding best Mie fit, is shown in Fig. 5(c). The mean size was found to be 10.01 µm with a standard deviation of 0.029 µm. These values were within the manufacturer’s specification for these microspheres of 10.00 µm ± 0.08 µm.
3.2 In vitro cell results

Fig. 6. Results of in vitro studies of each cell type. (a,b,g,h) Histogram plots of characteristic cell sizes extracted from the best Mie spectra fit with mean and standard deviation; (c,d,i,j) Representative spectrum of a single cell from each blood cell type, and its best Mie spectra fit; (e,f,k,l) OCT B-scan image of each cell sample with color-coded overlay of the characteristic best-fit sizes of individual cells observed (see color bar for best-fit cell size scale). Distance scale bar (white): 100µm.

Isolated RBCs (N = 634), granulocytes (N = 769), lymphocytes (N = 737) and monocytes (N = 638) in separate glass cuvettes were imaged. Individual cells were localized and tracked in either B-scan or volumetric imaging mode based on the cell moving speed evaluation criteria.
in Section 2.3, and the characteristic size of each tracked cell based on the best Mie spectra fit was found. Histogram plots of characteristic sizes of each cell type are shown in Fig. 6(a,b,g,h), along with their means and standard deviations. For RBC, the characteristic sizes (Fig. 6a) shows a bimodal distribution instead of a normal distribution; Therefore, we calculated the probability density function (PDF) of the RBC size distribution using kernel density estimation, and fitted the PDF with the following bimodal Gaussian distribution function,

\[
f(x) = a_1 e^{-\frac{(x-\mu_1)^2}{2\sigma_1^2}} + a_2 e^{-\frac{(x-\mu_2)^2}{2\sigma_2^2}}.
\]

The means and standard deviations of the best-fit bimodal distribution were found to be, \( \mu_1 = 1.94 \, \mu m, \mu_2 = 5.41 \, \mu m, \sigma_1 = 0.85 \, \mu m, \) and \( \sigma_2 = 0.98 \, \mu m. \) In Fig. 6(c,d,i,j), we show the extracted backscattering spectra, best-fit Mie spectra, and extracted characteristic size of a representative single cell from each cell type. In Fig. 6(e,f,k,l), we show the B-scan images containing the cells depicted in Fig. 6(c,d,i,j), with all cells in the B-scan color-coded to represent the best-fit characteristic sizes of each individual cells within the image. Note that cells within the B-scan without color coding (shown in gray) correspond to cells that did not remain in the FOV for at least 20 repeated B-scans or volumes.

A boxplot representation of the characteristic size distributions for each cell type is shown in Fig. 7. The means of the size distributions were all found to be significantly different between each pairs of cell types using one-way ANOVA and post hoc tests with the Bonferroni correction \( (p < 0.0001/6 = 1.66*10^{-5}), \) which indicates that characteristic size distribution could be used to differentiate between populations of RBCs and subtypes of WBCs. For patients with a dominant cell type present in the AC, the cell type can potentially be identified based on the extracted characteristic size distribution.

![Boxplot of characteristic size distributions](image)

**Fig. 7.** Boxplot of the characteristic size distributions of RBCs, granulocytes, lymphocytes and monocytes observed in the *in vitro* cell study. The box plot represents the range of characteristic sizes, with the red line corresponding to the median size of each cell type, the blue box corresponding to the interquartile distribution, the whiskers corresponding to the sizes within 1.5 times the interquartile range and the red plus signs representing outliers that are located outside the whiskers.

The size distribution of each type of cell was found to overlap with the size distributions of other types of cells, which means it will still be challenging to differentiate cell type at the single-cell level. To further quantify the overlap among the size distributions, the support vector machine (SVM) classifier with a Gaussian kernel and 10-fold cross validation was
applied, and the receiver operating characteristic (ROC) curves between the characteristic sizes of each pairs of cell types were plotted in Fig. 8, along with the corresponding areas under the curve (AUC).

![Fig. 8. The ROC curves between the characteristic sizes of each pairs of cell types, along with the corresponding AUC values.](image)

Although it remains challenging to differentiate cell types at the single-cell level, the more significant clinical target of this technology is likely to be in determination of true compositions of cell populations in the AC. In the case of a mixture of two or several types of blood cells without a significantly dominant cell type, the composition of the cell sample could be estimated by fitting the measured size distribution to a linear combination of reference size distributions of cells, which could be obtained from a large number of cell measurements. For example, the ratio of polymorphonuclear WBCs to mononuclear WBCs (mainly lymphocytes and monocytes) in uveitis patients could potentially be determined by fitting the measured size distribution to a linear combination of reference size distributions of these three types of cells.

### 3.3 Ex vivo cell results

We also investigated granulocytes injected into the ACs of freshly enucleated porcine eyes to mimic the in vivo environment. A representative OCT volume render with granulocytes suspended in the AC of a porcine eye is shown in Fig. 9(a). The characteristic size distributions of RBCs (N = 332), granulocytes (N = 246), lymphocytes (N = 268) and monocytes (N = 219) acquired from the porcine eye studies are shown in Fig. 9(b,c,f,g).
Fig. 9. Results of ex vivo porcine eye studies of each cell type. (a) Representative OCT volume of granulocytes suspended in the AC of a porcine eye; (b,c,f,g) Histogram plots of characteristic cell sizes extracted from the best Mie spectra fit with means and standard deviations; (d,e,h,i) Boxplot of the characteristic size distributions of all four types of cells in vitro and ex vivo, and the corresponding p-values calculated using the Wilcoxon rank sum test.

The mean characteristic cell sizes of the ex vivo porcine eye studies were very similar to the sizes observed in the in vitro studies for all four types of cells (RBC: 2.0 & 5.4 µm vs. 2.1 & 5.6 µm, granulocyte: 4.1 µm vs. 4.1 µm, lymphocyte: 5.7 µm vs. 5.7 µm, and monocyte: 6.9 µm vs. 6.8 µm). No statistically significant difference in the size distributions of all four types of cells between the in vitro and the ex vivo porcine eyes was found (Fig. 9(d,e,h,i)) using the Wilcoxon rank sum test (RBC: $p = 0.46$, granulocyte: $p = 0.76$, lymphocyte: $p = 0.58$, and monocyte: $p = 0.54$).

3.4 Red blood cell backscattering spectrum

The backscattering cross-section, with units of $\mu m^2$ or dB $\mu m^2$, indicates the scattering property of an object to reflect incident field energy back to the direction of incident field. The BOR-SIM theoretical backscattering spectra of a single RBC at four representative orientations with respect to incident light are shown in Fig. 10.
Fig. 10. The backscattering spectra of an RBC with different incident angles of light at two orthogonal linear polarizations. (a) 0°, perpendicular to the long axis of the RBC; (b) 30°; (c) 60°; (d) 90°, parallel to the long axis of the RBC. (e) The total backscattering cross-sectional intensity across the entire spectrum as a function of incident angle from 0° to 90° with a step size of 2.5°.

As expected, the frequency of oscillation features in the theoretical spectrum increases as the incident angle of field with respect to the RBC increases. The oscillation frequency of the spectrum is related to the axial optical path length of the scatterer. For an RBC, when the incident angle of field increases, the axial cross-section length increases from the short axis to the long axis, such that the spectrum has a higher oscillation frequency feature. The spectra at two orthogonal polarizations were completely overlapped when the incident angle of field was 0° (Fig. 10(a)), since the RBC is circular symmetric in the plane of the long axis. At other incident angles, the differences between spectra for the two orthogonal polarizations were minimal (Fig. 10(b)-(d)), and the different polarization spectra had minimal difference in frequency of oscillation features at each angle. Therefore, for simplicity we only used the theoretical spectra with horizontal polarization ($\theta$) for the further analysis below. More importantly, the minimal spectra difference supports that the polarization state of incident light on the sample need not be precisely controlled. Furthermore, the total backscattering cross-sectional intensity of a scatter can be obtained by integrating the intensity at each wavelength, and should be directly correlated to the intensity of a scatterer within an OCT A-scan. For RBCs, we applied this concept and calculated the backscattering cross-sectional intensity across the entire spectrum at each incident angle (0° to 90°) and plotted intensity as a function of wavelength (Fig. 10(e)). The magnitude of the backscatter cross section clearly
decreases with increasing incident field angle, as expected from the profile of the RBC cross section viewed as a function of obliquity.

We compared the BoR-SIM theoretical backscattering spectra and our Mie theory library (which assumes spherical scatterers) to extract characteristic sizes from model RBCs as a function of the incident light angle. The best-fit Mie spectra and the corresponding sizes at representative incident angles of light are shown in Fig. 11(a). The extracted sizes from best-fit Mie spectra are shown in Fig. 11(b) as a function of incident angles from 0° to 90°. When the oscillation frequency of a backscattering spectrum increased, the characteristic size extracted from the best-fit Mie spectrum also increased. The characteristic size range of our RBC spectra model was 1.5–8 µm diameter, which corresponds well to our experiment results, 1–7.8 µm diameter, shown in Fig. 6. When the incident angle of light was within the range of 0° to 30°, the extracted characteristic sizes were between 1.5 and 3 µm, which includes the length of the short axis of the RBC model, 2.5 µm. When the incident angle of light was increased to more than 50°, the extracted characteristic sizes rapidly increased to between 6 and 8 µm, which includes the length of the long axis of the RBC model of 7.8 µm. Thus, although the spherical model assumed by Mie theory is not an accurate morphological model for RBCs, the extracted size using Mie theory was found to correlate well to the axial cross-section length of the RBC at different orientations. These theoretical models, in general, agree with our observed experimental characteristic size distributions, which is a bimodal distribution with two means at approximately 2 µm and 5.4 µm diameter, shown in Fig. 6. However, for the theoretical model, the characteristic size averaged over all incident angles was 4.92 µm ± 1.93 µm and was larger than our experimental results of 3.35 µm ± 1.83 µm.

We also plotted the backscattering cross-sectional intensity as a function of characteristic size in Fig. 11(c). As expected, when the RBC had a small characteristic size, indicating that the incident angle of light was small, it had a higher backscattering intensity due to a larger cross-sectional area perpendicular to the incident field.

We compared this simulated result with our experimental results. The averaged peak intensity of individual RBCs in repeated OCT volumes was calculated, and the scatter plot of average intensities of individual RBCs (N = 133) vs. their characteristic sizes is shown in Fig. 12(a). Representative spectra from two individual RBCs and their best-fit Mie spectra are shown in Fig. 12(b)-(c). We observed a similar, negative correlation between RBC
backscattering intensity and its characteristic size: a RBC with a higher backscattering intensity tends to have a smaller characteristic size. This unique negative correlation may provide an additional dimension of data to differentiate RBCs from WBCs. For WBC nuclei, which are more spherical, the backscattering intensity is expected to increase as the size of the cell nucleus increases.

![Fig. 12. (a) The intensity of individual RBCs in the OCT volume vs. their characteristic sizes. (b)-(c) Two representative spectra from RBCs with small and large characteristic sizes.]

Additionally, we noticed intensities of some individual RBCs were only slightly above the intensity threshold used to locate and track each cell. This threshold value was previously optimized to maximize the number of localized cells while not labeling noise within the OCT image as individual cells. Thus, some cells with intensities below the threshold were likely excluded from the analysis, and those cells were more likely to have larger characteristic sizes, based on our simulation results, as seen in Fig. 11(c). This may be the primary cause of the differences between the smaller mean characteristics size of our experimental results, 3.35 µm, and the larger mean characteristic size of our RBC theoretical model, 4.92 µm. Another potential source of error could be the geometry model and the inhomogeneous refractive index distribution of RBC.

Overall, we demonstrated that although Mie theory is not an accurate model for calculating the physical size of RBCs due to their nonsphericity, the extracted size from Mie theory fit was highly correlated to the axial cross-section length of RBC at each incident angle of light, which explains why their characteristic size distribution extracted from Mie theory was sufficient to statistically differentiate populations of RBCs from WBCs.

4. Discussion

We have shown that spectroscopic OCT analysis of single blood cells can differentiate populations of RBCs and subtypes of WBCs, including granulocytes, lymphocytes and monocytes. The characteristic size distribution extracted from the Mie theory fit was found to be significantly different among these different types of blood cells. Previous studies have shown that the characteristic sizes extracted from WBCs using Mie theory are likely to be their nuclear sizes for mononuclear leukocytes such as lymphocytes and monocytes, and sizes of the lobules of the nucleus for polymorphonuclear leukocytes such as neutrophils [32]. There was generally good agreement between our results and the nuclear sizes acquired from previous studies using conventional optical microscopy [43], differential interference contrast microscopy [32], and light-scattering spectroscopy [32]. For the granulocytes, mainly neutrophils, the reported mean characteristic size, 4.1 µm, was consistent with the nuclear lobe sizes reported from other studies, which ranged from 3.0 to 4.4 µm. For lymphocytes, the mean size of 5.7 µm was also within the range of previous reported values, which was from 4.5 µm to 6.4 µm. Three-dimensional refractive index maps of subtypes of WBCs were
previously studied using refractive index tomography [33,34]. However, no quantitative information regarding the nucleus sizes were reported directly in these studies. Moreover, a Mie theory approach here assumes nuclei or nuclei lobes that are spherical, of a uniform refractive index, and that the backscattering signal is dominated by the scattering from the nuclei, all of which oversimplifies the complex morphology of cells. For instance, monocytes tend to have a kidney-bean shaped nucleus while the nuclei of neutrophils have lobes which may not be appropriately modeled as spherical [44]. Additionally, the refractive index we used for our Mie theory library was measured using refractive index tomography at 532nm and 633nm, as there have been no studies at 1000-1100nm wavelength range. However, as demonstrated in this paper, Mie theory is a sufficient model to statistically differentiate populations of subtypes of WBCs.

In this work, we have developed an algorithm to track single cells in repeated B-scans or volumes. Our scanning protocols were selected based on the moving patterns of \textit{in vitro} and \textit{ex vivo} cell samples, which were heavily affected by the gravity. For future \textit{in vivo} studies, these protocols will likely need to be adjusted, because cells in the AC are known to follow a circulating flow pattern due to the natural temperature gradient [25], and living human eye motion artifacts will need to be mitigated. Multiple techniques could be utilized to reduce significant artifacts due to motion during \textit{in vivo} imaging. First, a swept source with a much faster A-scan rate can be used to reduce the acquisition time. For example, with a 1MHz swept source, one acquisition of our repeated B-scan mode (500 A-scans/B-scan, 50 B-scans) would take only 40ms. Another approach could be to use repeated B-scans or volumetric scans with a smaller FOV but a higher effective frame rate. Although fewer cells would be localized in a single acquisition, our cell tracking algorithm would be less likely to be affected in a shorter acquisition time. Multiple measurements could be performed at different locations of the AC to achieve a similar total number of cell measurements. Other real-time eye tracking and motion compensation techniques may also be considered, such as pupil tracking using a 2D fast steering mirror [45] and SLO-based eye tracking [46].

Our porcine eye studies demonstrated that OCT signal attenuation in even deceased corneas did not compromise our spectroscopic analysis methodology. Given that porcine corneas are thicker than human corneas, and that \textit{ex vivo} corneas lack the cellular processes which actively maintain corneal clarity \textit{in vivo}, we believe the porcine corneas were a fair test of robustness with regard to SNR. Finally, we note that as described in the method section 2.1, our illumination power on the sample in both \textit{in vitro} and \textit{ex vivo} studies was 2.2mW, which is well below the optical power safety limit of the newer ANSI standard (Light Hazard Protection for Ophthalmic Instruments - ANSI Z80.36-2016).

The lateral resolution of our system was 20 \(\mu\)m, so that the system had a large enough depth of focus to localize individual cells across the whole AC depth. This resolution is similar to the lateral resolution of conventional, clinical anterior segment OCT systems, which indicate that this proposed spectroscopic technique could potentially be applied directly to widespread clinical OCT systems. However, a higher resolution system may be considered for future \textit{in vivo} imaging to improve the backscattering signal from the cell, but with a tradeoff of the depth of focus. Finding the optimal balance between depth of focus and lateral resolution for our technology is important for our future \textit{in vivo} studies.

In both \textit{in vitro} and \textit{ex vivo} experiments, the cell samples were diluted to a concentration of \(\sim150\) cells/mm\(^3\) and minimal cell aggregations were observed. For patients with inflammatory cellular response in the AC, such as anterior uveitis patients, the number of cells is assessed by the examiner in a slit-lamp microscope evaluation and assigned an integer score from 0 (no cells seen) to 4 + (many cells). The highest clinical grade of 4 + corresponds to \(\geq50\) cells in a lateral FOV of 1 mm *1mm [47]. Given that the average human anterior chamber depth is approximately 3mm [48], the cell concentration of a uveitis patient with a slit-lamp grade of 4 + is thus \(\geq17\) cells/mm\(^3\), which is much smaller than the concentration we used in our \textit{in vitro} and \textit{ex vivo} studies. Moreover, prior studies from Rose-Nussbaumer et al.
also showed that the average in vivo cell axial size from uveitis subjects were only slightly larger than in vitro cell size measurements from OCT images (in vivo 6.7–6.8 versus in vitro 6.3 μm), which also suggests that aggregated cells are not common in in vivo imaging [17].

The methodology demonstrated here could be applied to investigate cell compositions in the vitreous body in several diseases, such as posterior uveitis, using a retinal OCT scanner. Without any required hardware modifications, this proposed spectroscopic technique could potentially be applied as a software upgrade to widespread clinical OCT systems, and combined with other cell information that may further facilitate cell differentiation, such as flow speed, intensity and spatial distribution of cells. Machine learning based approaches could also be implemented to improve the classification.

5. Conclusion

In this paper, we developed a spectroscopic OCT analysis method to differentiate between RBCs and subtypes of WBCs, including granulocytes, lymphocytes and monocytes. We located and tracked individual blood cells in repeated OCT B-scans or volumes, and extracted their corresponding backscatter spectra using spectroscopic OCT. We then correlated the spectrum of each cell to its characteristic size by fitting with a Mie theory database. Together, our in vitro studies indicated that the extracted size distributions based on the best Mie spectra fit were significantly different between each cell type. To confirm these observations, our pilot ex vivo studies with blood cells injected into porcine eyes suggested that spectroscopic OCT can potentially differentiate blood cells in AC in vivo. We further studied the backscattering spectrum of RBC at different incident directions of light. We showed that, although Mie theory is not an accurate model for RBCs, the extracted size from Mie theory was highly correlated to the axial cross-section length of RBC at different orientations, sufficient to differentiate RBCs from WBCs. The above described work can potentially provide quantitative diagnostic information of cellular responses in the ocular anterior chambers of patients in the clinic and expand the field’s pathophysiologic knowledge about diseases such as anterior uveitis.

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