DEVELOPMENT OF A NEW SPECTROSCOPIC METRIC FOR SCATTERER SIZE ESTIMATION USING OPTICAL COHERENCE TOMOGRAPHY (OCT)

by

Michalis Kassinopoulos

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Michalis Kassinopoulos

Examination Committee:

Dr. Constantinos Pitris
Associate Professor, Department of ECE, Research Supervisor

Dr. Georgios Ellinas
Associate Professor, Department of ECE, Committee Member

Dr. Christina Orphanidou
Visiting Lecturer, Department of ECE, Committee Member
Abstract

In this thesis, we describe a new metric for spectroscopic Optical Coherence Tomography (SOCT) that is found to enhance the contrast in OCT images based on the distribution of scattering particle sizes. The basic idea behind this metric is that the degree of modulations appeared in the backscattering spectrum of a scatterer is strongly related to its diameter. Even though many other metrics have been proposed sharing the same idea, none of them has achieved a high sensitivity to scatterer size. In this study, we use Mie theory scattering to do some further investigations related to the relationship between the degree of modulations in the spectrum and the diameter in order to better understand the dependence of this relationship on the spectral range of the light source and the medium and scatterer refractive indices, as also to understand the limitations of a metric based on this relationship. Moreover, we discuss the importance of the relative size and position between a scatterer and a window in the spatial domain in order to obtain a representative backscattering profile. Finally, we demonstrate the feasibility of our approach for contrast enhancement in phantom samples of 6, 10 and 16 μm microspheres. The results are very encouraging, suggesting that the proposed metric could be implemented in OCT spectral analysis for measuring nuclear size distribution in biological tissues. A technique providing such information would be of great clinical significant since it would allow the detection of nuclear enlargements at the earliest stages of precancerous development.
Περίληψη

Στη παρούσα διπλωματική εργασία, περιγράφουμε ένα νέο δείκτη για φασματοσκοπική Οπτική Τομογραφία Συνοχής - OCT (Optical Coherence Tomography) ο οποίος δείχνει να αυξάνει την αντίθεση σε εικόνες OCT βάση της κατανομής των διαστάσεων των σκεδαζόμενων σωματιδίων. Η βασική ιδέα πίσω από αυτόν τον δείκτη είναι το ότι ο βαθμός διακυμάνσεων που εμφανίζεται σε ένα οπισθοσκεδαζόμενο φάσμα ενός σκεδαστή έχει σημαντική συσχέτιση με την διάμετρο του. Αρκετοί δείκτες που βρίσκομαι στην βιβλιογραφία βασίζονται στην ιδέα αυτή. Παρόλα αυτά, κανένας από αυτούς τους δείκτες δεν έχει μεγάλη ευαισθησία στη διάμετρο του σκεδαστή. Σε αυτή την εργασία, χρησιμοποιούμε την θεωρία του Mie που αναφέρεται στη σκέδαση του φωτός πάνω σε σωματίδια, για να κάνουμε κάποιες διερευνήσεις ώστε να καταλάβουμε καλύτερα την εξάρτηση αυτών των διαστάσεων και πως αυτή η σχέση επηρεάζεται από το εύρος των πηγών και τους δείκτες διάθλασης του σκεδαστή και του μέσου που βρίσκεται. Επίσης, κάνουμε τις διερευνήσεις αυτές για να γνωρίζουμε καλύτερα τους περιορισμούς που έχει ένας δείκτης βασισμένος στην ιδέα αυτή. Επιπρόσθετα, συζητούμε την διερεύνησή του σχετικού μεγέθους και της σχετικής θέσης μεταξύ του σκεδαστή και του παραθύρου στο πεδίο του χώρου (spatial domain) έτσι ώστε να γνωρίζουμε πως μπορούμε να πάρουμε έναν αντιπροσωπευτικό οπισθοσκεδαζόμενο προφίλ. Τέλος, παρουσιάζουμε δείγματα OCT δειγμάτων με 6, 10 και 16 μμ μικροσφαιρίδια όπου φαίνεται καθαρά η δυνατότητα της μεθόδου που προτείνουμε για αύξηση της αντίθεσης στην εικόνα. Τα αποτελέσματα της εργασίας μας είναι πολύ ενθαρρυντικά και φανερούν τις προοπτικές του δείκτη σε περίπτωση που ενσωματωθεί στη φασματική ανάλυση OCT, για μέτρηση της κατανομής του μεγέθους των πυρήνων σε
βιολογικούς ιστούς. Αν μια τέτοια τεχνική υλοποιηθεί που να παρέχει την συγκεκριμένη πληροφορία, αυτό θα ήταν μεγάλης σημασίας καθώς θα επέτρεπε την έγκαιρη ανίχνευση των αλλαγών στους πυρήνες στα αρχικά στάδια της προκαρκινικής ανάπτυξης.
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Michalis Kassinopoulos
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1 Introduction

Optical coherence tomography (OCT) is a non-invasive, high-resolution imaging technique capable of producing in vivo cross-sectional images of subsurface microstructure of biological specimens (Huang et al. 1991). The optical configuration of OCT is typically consisted by a Michelson interferometer and a broadband light source which enables the detection of echo time delay of backscattered and backreflected optical signals from different layers within the sample. The light source of an OCT system operates at the optical window, also known as the therapeutic or NIR window, in which biological tissues have less absorption and scattering and the maximum penetration depth of light into tissue is achieved. Moreover, because of the use of NIR, OCT has a minor radiation exposure compared with the other techniques of tomographic imaging (e.g. CT and μCT). As an optical technique, OCT benefits from a simple and flexible design, facilitating exchanges and upgrades, enabling to contribute to many fields in medicine such like in ophthalmology, gastroenterology, dermatology and cardiology.

A research that is going on the latest decades in the field of light scattering spectroscopy and attracts also interest in the OCT research community is the development of a method to detect morphological changes in epithelial cells. 90% of all human cancers arise in the epithelial tissue and if the lesions are diagnosed in one of the preinvasive stages they are readily treatable. However, early lesions are often almost impossible to detect visually. The conventional method of detecting this changes is biopsy. A small fraction of the epithelial surface is excised from the tissue and a pathologist examines it under the microscopic, but such an approach has several drawbacks including the subjectivity of diagnoses, the inherent invasiveness of biopsies, the time delay between biopsy and diagnosis, and the poor coverage of at-risk tissue.

Adler et al. first reported a spectrometric for single-particle systems that measures the degree of modulation on the backscattered spectrum which is correlated to the particle size. This metric is based on the bandwidth of the autocorrelation function (ACF) of the depth-resolved
spectra. By encoding the values of this metric in the color map of an OCT image, contrast enhancement can be achieved (Adler et al. 2004). Since then, many studies have been done with variations of this method, experimenting in artificial microspheres and cellular monolayers. Tay et al. proposed an extended version of the ACF metric which, as far as we know, yields the highest sensitivity in scatterer size compared to other metrics in the literature (Tay et al. 2012). The feasibility of their metric to differentiate scatterers of different sizes was demonstrated in SOCT images of 0.5 and 45 μm microsphere solutions in which the two control microsphere samples could clearly be distinguished.

In this thesis, we propose an extended version of the ACF metric that is based on the autocorrelation of the first derivative (COD) of the backscattering spectrum and shows to be more sensitive than other metrics in previous works. Chapter 2, is a review of the fundamental principles and theory behind OCT as well as of the contribution of light scattering spectroscopy (LSS) and OCT in nuclear morphology measurements. Next, Chapter 3, determines the COD bandwidth metric that we propose in this thesis, and then it describes the procedure we followed to create the microsphere phantom samples as also the different steps involved in the spectral analysis of the OCT images. In Chapter 4, the SOCT images of 6, 10 and 16 μm microsphere samples are presented along with statistics referred to the diameter estimations. Finally, Chapter 5 and Chapter 6 describe the conclusions that we reached from carrying out this study and provide suggestions for future work.
2 Literature Review

2.1 Optical Coherence Tomography

2.1.1 Development of Optical Coherence Tomography (OCT)

Optical coherence tomography (OCT) is a high-resolution, interferometric imaging modality that uses near-infrared light to obtain cross-sections and three-dimensional images of internal microstructures in biological systems. Imaging can be performed in vivo and in real time providing images with resolution of 1 to 15 μm. OCT is often compared to ultrasound imaging since these two modalities work in a similar way. In both modalities there is a probe beam directed onto the biological specimen and a detector that receives the reflections from the sample. This signal is then analyzed in order to obtain depth information. However, in ultrasound, the depth of tissue structures is determined by directly measuring the time delay of the returning signal whereas in OCT, methods of low-coherence interferometry are employed so as to measure the time delay. Thus, OCT images reflect the optical backscattering profile of the sample which is used to derive its structural information.

One of the first applications of interference in biomedicine was so-called retinometry and it was introduced in 1935 by Yves Le Grand (Flammer et al. 2013). Later, in 1986, two new optical techniques were demonstrated for the measurement of intraocular distances. Fujimoto et al demonstrated a technique that uses femtosecond laser pulses to measure the cornea in rabbit eyes in vivo (Fujimoto et al. 1986), whereas Fercher and Roth reported the applicability of low coherence interferometry to measure the optical length of human eyes in vivo (Fercher & Roth 1986). The term “Optical Coherence Tomography” was coined in 1991 by Fujimoto’s and Huang’s team (Huang et al. 1991) when they reported an extension of low-coherence reflectometry to tomographic imaging. The OCT system they developed could perform multiple longitudinal scans at a series of lateral locations providing a two-dimensional map of reflection sites in the biological system that is been examined. Tomographic imaging is also demonstrated in their work derived from the peripapillary area of the retina and the
Optical Coherence Tomography

Since then, OCT has become an essential diagnostic technique in clinical ophthalmology (Figure 2.1).

At the same time, technological advances in laser sources, beam delivery systems, and detection schemes, allowed OCT to enter many new clinical areas such as gastroenterology, dermatology, cardiology, and oncology (Zysk et al. 2007). High-speed imaging capabilities are some of the most important advances, as they have enabled higher frame rates and thus reduction of artifacts due to patient motion. Moreover, these capabilities allowed the construction of 3-D volumetric images at shorter time periods. OCT imaging speed has been improved significantly with the development of Fourier domain detection techniques such as spectral-domain OCT (SD-OCT) and swept-source OCT (SS-OCT), where rapid scanning of narrow-band source spectra is performed. Finally, since OCT penetration depths are typically limited to about 1 to 3 mm in highly scattering tissues, a variety of imaging devices have been integrated into clinical devices to allow for minimally invasive OCT imaging throughout the body with endoscopes, catheters, and biopsy needles (Zysk et al. 2007).

Figure 2.2 illustrates the number of academic journal publications per year submitted in the PubMed database that have “Optical Coherence Tomography” in the title for the period 1991-2014. As can be seen, after the year 2000 there is a linear increase in the annual number of publications related to OCT illustrating the active and rapidly expanding OCT community.
Literature Review

OCT system operates at the optical window 700-1400 nm (also known as the therapeutic or NIR window), in which biological tissue has less absorption and scattering, improving in that way the penetration depth of light into tissue. Since most of the incident photons are transmitted and absorbed, only a small part of ballistic and quasi-ballistic photons carry information about the sample, resulting in a maximum imaging depth of approximately 2-3 mm (Walther et al. 2011). The largest advantage for working at the NIR range is the minor radiation exposure compared to other modalities for tomographic imaging such as computer tomography (CT) and X-ray microtomography (μCT). Compared to conventional confocal microscopy, OCT has larger penetration depth and higher frame rates, especially for 3-D images. Additionally, OCT, as an optical technique, provides images with much higher resolution than those obtained by the use of ultrasound (US), CT and magnetic resonance tomography (MRT). Last, some other main advantages of OCT are the noninvasive and contactless application, the relatively low cost, the robustness, and the transportability of most OCT systems.

Figure 2.2: Number of academic journal publications per year with “Optical Coherence Tomography” in the title as indexed in the PubMed database between 1991 and 2014.
2.1.2 Instrumentation of OCT

OCT relies on low-coherence interferometry (LCI) in which the interference pattern is analyzed and the intensity of the backscattered signal as a function of depth is obtained. One of its advantages as an interferometric technique is its superior signal-to-noise ratio (SNR) which can reach values greater than 100 dB (Walther et al. 2011). Although bulk optics can be used for OCT systems, people tend to use single-mode fibers which are much more flexible and can be combined with a large variety of available fiber optic components. An OCT system typically comprises a Michelson-type interferometer in which the fiber optic variant consists of a 2x2 fiber coupler with the reference and the sample beam in the two arms opposite to those of the light source and detection (Figure 2.3).

![Figure 2.3: Schematic diagram of a time domain OCT system. The light from an SLD is split into sample and reference arm by a fiber coupler (FC). While the beam is reflected by a moving mirror in the reference arm, in the sample arm part of the light will be reflected from different structures of the sample. The interfering light is focused on a detector. L1–L3 are different lenses or objectives focusing the beam. Each reflection from the sample results in a burst of the detector signal at the corresponding position, which is demodulated by the electronics (Walther et al. 2011).]
The range of the light source used in an OCT system, combined with sample’s optical properties, determines the maximum penetration depth and the axial (depth) resolution. As mentioned before, the OCT system operates at the optical window (700-1400 nm) where biological tissues have relatively high transmission. The lower and upper limits of this window are due to absorption of light by blood and water respectively. In OCT imaging there is always a trade-off between resolution and penetration depth. Specifically, enhanced penetration depth is observed for wavelengths in the region of 1300 nm, because of less tissue scattering, whereas imaging at shorter wavelengths, for example in the region of 800 nm, is characterized by higher resolution.

Axial (depth) resolution is an important specification of an OCT system. In many biomedical applications high axial resolutions are required to distinguish cellular boundaries and types. In OCT related literature the system axial resolution is generally defined as half the source coherence length $l_c$. The coherence length can be defined in a number of ways, the most commonly quoted of these in OCT is the full-width at half maximum (FWHM) of the source self-coherence function (SCF) multiplied by the speed of light. The SCF is simply the inverse Fourier transform of the source intensity spectrum. Assuming a Gaussian shaped spectrum of width $\Delta \lambda$ and center wavelength $\lambda_0$, the coherence length is determined by

$$l_c = \frac{4 \ln(2) \lambda_0^2}{\Delta \lambda} \approx 0.88 \frac{\lambda_0^2}{\Delta \lambda}.$$  

Therefore, the theoretical OCT resolution $\mathcal{R}_{OCT}$ is given by

$$\mathcal{R}_{OCT} = \frac{l_c}{2} \approx 0.44 \frac{\lambda_0^2}{\Delta \lambda}.$$  

The large bandwidth of some light sources, in conjunction with the fact that the resolution of OCT in a medium of refractive index $n$ is $l_c/(2n)$, enables resolution in tissue of approximately 1 $\mu$m (Drexler et al. 1999). Although systems based on femtosecond lasers may achieve higher resolution, superluminescent diodes (SLD) are the best choice in respect of size, price, and ease of operation (Ko et al. 2004). The spectral width of a single SLD,
which is in the range 25 to 75 nm, enables axial resolution well below 10 μm at wavelengths in the 800 nm region and approximately 10 μm at wavelengths in the 1300 nm region.

In the next two sections, the two different technical approaches used to acquire depth scans in OCT are explained.

**Time-domain OCT**

The first generation of OCT systems, denoted “time-domain OCT” (TD-OCT), uses a broadband light source and a single detector at the output of the interferometer; a fiber optic variant is shown in Figure 2.3. By changing the length of the reference beam, the light backscattered from within the sample is analyzed in respect to the optical pathway. Because interference of a broadband wavelength spectrum is only detected when both arms of the interferometer have a difference in length shorter than the coherence length $l_c$ of the light source, the origin of the back-reflected sample light can be determined accurately. Sharp refractive index variations between layers in the sample manifest themselves as corresponding intensity peaks in the interference pattern. A time-domain interference pattern can be obtained by translating the reference mirror to change the reference path length and match multiple optical paths due to layer reflections within the sample. The capture of this interference is called depth scan or A-scan, by analogy with ultrasound.

Moving the beam laterally relative to the sample in one dimension in order to obtain a cross-sectional view and coding the signal strength to the brightness in the resulting image is referred to as a B-scan. To obtain volumetric information about the sample, the beam is deflected in two dimensions relative to the sample surface. Scanning can be achieved by moving the sample, although deflection of the beam with one or two galvanometer scanners is preferred for high-speed imaging. The 2-D and 3-D information for generating B-scans or volume scans can also be acquired by illuminating a larger area of the sample and mapping the interference light onto a line or array detector, respectively. Although such TD OCT systems achieve a very high resolution in all dimensions, they are limited in imaging speed (A-scans per time) by the mechanical movement of the translation stage.
Fourier-domain OCT

Despite all efforts to increase the speed of optical delay lines in TD-OCT, the breakthrough in high-speed OCT imaging was achieved by introducing the concept of Fourier domain OCT (FD-OCT) which is also known as “spectral interferometry” (Fercher et al. 1995), “coherence radar”, or “spectral radar” (Bail et al. 1996; Häusler & Lindner 1998). FD-OCT is based on the fact that each wavelength of the light in the Michelson interferometer will interfere even if the reference and sample arms have totally different lengths. For a fixed difference in length between both arms, the interfering light will oscillate as a function of the wave number with a frequency that is proportional to the length difference. Therefore, Fourier transformation of the spectrum yields the backscattering amplitude as a function of depth. Generally, the

![Diagram of an SD-OCT system](image)

**Figure 2.4:** Schematic diagram of an SD-OCT system. Different from TD-OCT, the mirror in the reference arm is stationary. The interfering light from the fiber coupler (FC) is spectrally resolved by the grating (G) and focused on the line-scan camera. L1–L4 are different lenses or objectives. The camera detects the interference spectrum. After Fourier transformation the depth dependent signal is recovered (Walther et al. 2011).
interference spectrum is acquired in two ways, first by analyzing the interfering signal with a spectrometer which is referred to as spectral domain OCT (SD-OCT) (Figure 2.4), or, second, by sweeping the wavelength of the light directed to the interferometer as a function of time (Figure 2.5). The latter is denoted as swept source OCT (SS-OCT) or optical frequency domain imaging (OFDI). The imaging speed of FD-OCT systems is only limited by the readout rate of the line-scan camera of the spectrometer or the tuning velocity of the laser. Compared with TD OCT, the SNR achievable in the same time with the same amount of light is larger by a factor scaling with the square root of the number of depth data to be acquired (Leitgeb et al. 2003; Choma et al. 2003; Yun et al. 2003). Today, spectrometer-based OCT systems achieve velocities of more than 200,000 A-scans per second. By
introducing new concepts of swept sources, SS-OCT systems have acquired even more than 20 million A-scans per second (Wieser et al. 2010).

2.1.3 Theoretical formulation

To describe OCT mathematically it is useful to express the electric field $E(\omega, t)$ as a complex exponential (Tomlins & Wang 2005):

$$E(\omega, t) = s(\omega)e^{-i(\omega t + kz)}.$$  (3)

This is a plane polarized solution to the wave-equation, with source field amplitude spectrum $s(\omega)$, frequency $\omega$ and time variation $t$. The second term in the exponential, in terms of wavenumber $k$ and distance $z$, simply accounts for phase accumulated throughout the interferometer. Since the input phase is arbitrary, and the interferometer only measures the relative output phase between the two optical paths, the phase term can be dropped from the input electric field. The field in each part of the interferometer is denoted by subscripts as follows; $E_{in}$, $E_{out}$, $E_r$ and $E_s$, corresponding to optical fields in the input, output, reference and sample arms, respectively (Figure 2.6). The reference mirror is assumed to be ideal and

Figure 2.6: Basic OCT system, based on a Michelson interferometer (Tomlins & Wang 2005)
the beam-splitter has reference and sample arm intensity transmittance $T_r$ and $T_s$ respectively. The intensity transmission coefficients are related, such that $T_r + T_s = 1$. The sample has a frequency domain response function $H(\omega)$ that describes its internal structure and accounts for phase accumulation therein. Therefore, the component optical fields are given in terms of the input field:

$$E_{in}(\omega, t) = s(\omega) e^{-i(\omega t)}$$  \hspace{1cm} (4)

$$E_r(\omega, t, \Delta z) = \sqrt{T_r T_s} E_{in}(\omega, t) e^{-i\varphi(\Delta z)}$$  \hspace{1cm} (5)

$$E_s(\omega, t) = \sqrt{T_r T_s} E_{in}(\omega, t) H(\omega),$$  \hspace{1cm} (6)

$$E_{out}(\omega, t, \Delta z) = E_r(\omega, t, \Delta z) + E_s(\omega, t),$$  \hspace{1cm} (7)

where $\varphi(\Delta z)$ is the phase accumulated in translating the reference mirror by a geometric distance $\Delta z = \Delta t \left(\frac{c}{n_{air}}\right)$.

$$\varphi(\Delta z) = \frac{2\omega n_{air} \Delta z}{c}$$  \hspace{1cm} (8)

$\Delta t$ is the corresponding optical time of flight difference and as usual, $c$ represents the speed of light in vacuum. $n_{air}$ is the group refractive index of air. The factor of 2 arises because of the Michelson interferometer configuration, where the path length change is always double the distance that the reference mirror is displaced. Clearly, these equations assume that the interferometer is being operated in air. It is also noted that the frequency domain product of the sample response function and input field is equivalent to the convolution of the response function with the input field in the time domain (Hariharan 2003).

Optical detectors are square law intensity detection devices, where the recorded intensity is proportional to a time average over the electric field multiplied by its complex conjugate:

$$I(\omega, \Delta z) = \langle E_{out}(\omega, t, \Delta z) E_{out}^*(\omega, t, \Delta z) \rangle$$  \hspace{1cm} (9)
The angled brackets denote a time-average, given by

\[
I(\omega, \Delta z) = \lim_{T \to \infty} \frac{1}{2T} \int_{-T}^{T} E_{\text{out}}^{*}(\omega, t, \Delta z)E_{\text{out}}(\omega, t, \Delta z)dt
\]  

(10)

Substituting equation (7) into equation (9), it can be shown that the intensity is a sum of three terms:

\[
I(\omega, \Delta z) = \langle E_{s}E_{s}^{*} \rangle + \langle E_{r}E_{r}^{*} \rangle + 2\Re\{\langle E_{s}E_{r}^{*} \rangle\}.
\]  

(11)

The first two terms can be identified as “self-interference”, whereas the last term is the real part (denoted \(\Re\) ) of the complex “cross-interference”. Making the relevant substitutions from equations (4)–(6) and substituting for the field spectrum \(s(\omega)\) a corresponding intensity spectrum \(S(\omega) = |s(\omega)|^{2}\), the frequency and path difference dependent intensity is given by

\[
I(\omega, \Delta z) = T_{r}T_{s} S(\omega)|H(\omega)|^{2} + T_{r}T_{s} S(\omega) + 2T_{r}T_{s} \Re\{S(\omega)H(\omega)e^{-i\phi(\Delta z)}\}.
\]  

(12)

The sample response function \(H(\omega)\) describes the overall reflection from all structures distributed in the \(z\) direction within the sample, and is given by

\[
H(\omega) = \int_{-\infty}^{+\infty} r(\omega, z)e^{-i2n_{g}(\omega, z)\omega z/c}dz.
\]  

(13)

The function \(r(\omega, z)\) is the backscattering co-efficient from the sample structural features, \(n_{g}(\omega, z)\) is the frequency dependent, depth varying group refractive index given by

\[
n_{g}(\omega, z) = \int_{0}^{z} n(\omega, z')dz',
\]  

(14)
and the exponential term accounts for phase accumulated by the multiple optical paths within the sample.

From equation (12) it is evident that information about the optical structure of the sample can be obtained from measurements in both the time and frequency domains. These two OCT modalities, are discussed below.

**Equation of the time-domain OCT interference pattern**

As described before, the system where a reference mirror is scanned to match the optical path from reflections within the sample is called time-domain OCT (TD-OCT). Equation (12) can be written as a function of reference path length displacement by integrating over the source spectrum. The expression is further simplified by assuming that the beam-splitter is lossless and has an ideal 50 : 50 split ratio, i.e. the transmissivity $T_r = T_s = 0.5$. Therefore, the TD-OCT interference pattern obtained in each axial scan is given as the sum of two terms

$$I(\Delta z) = I_0 + \Re\{\Gamma(\Delta z)\}. \tag{15}$$

$I_0$ includes only the contribution from self-interference:

$$I_0 = \frac{1}{4} \int_{-\infty}^{+\infty} S(\omega)(|H(\omega)|^2 + 1) d\omega \tag{16}$$

and $\Gamma(\Delta z)$ has only a contribution from the cross interference:

$$\Gamma(\Delta z) = \frac{1}{2} \int_{-\infty}^{+\infty} H(\omega)S(\omega)\cos\{\varphi(\Delta z)\} d\omega. \tag{17}$$

A simple layered sample can be modelled by writing the continuous sample integral, equation (13), as a summation over $N$ individual layers and assuming negligible dispersion:
\[
H = \sum_{j=1}^{N} r_j e^{i \frac{\omega}{c} \sum_{m=1}^{j} n_{g,m} z_m}.
\]

(18)

Here, \(z_m\) is the thickness of the \(m^{th}\) layer, with a group refractive index \(n_{g,m}\). The reflectivity of each layer, \(r_j\), can be determined by application of Fresnel’s equations (Yeh 2005). Assuming that the light is perpendicular to each layer, the reflectance is

\[
r_j = \frac{n_{j+1} - n_j}{n_{j+1} + n_j}.
\]

(19)

A typical TD-OCT system might use a superluminescent diode (SLD) source operating with a center wavelength \(\lambda_0 \approx 800 \text{ nm}\) and spectral FWHM of around 50 nm. In such a system, the axial resolution would be approximately 6 \(\mu\)m. Some refractive index and thickness values are given in Table 1 for a theoretical layered sample. Using these values, the resulting interferogram is plotted in Figure 2.7 as a function of the reference mirror displacement.

<table>
<thead>
<tr>
<th>Layer j</th>
<th>Refractive index (n)</th>
<th>Layer thickness (z) ((\mu)m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>5.00</td>
</tr>
<tr>
<td>2</td>
<td>1.30</td>
<td>15.00</td>
</tr>
<tr>
<td>3</td>
<td>1.50</td>
<td>30.00</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 1: Theoretical sample layer properties.
Equation of the Fourier-domain OCT interference pattern

Fourier domain OCT (FD-OCT) (Fercher et al. 1995) has the advantage that no moving parts are required to obtain axial scans. For the case of a SD-OCT the reference path length is fixed and the detection system is replaced with a spectrometer (Figure 2.4) whereas for an SS-OCT system the source is replaced with a swept source (Figure 2.5) and the obtained time-dependent data is first interpolated to the frequency domain before applying the following analysis. The detected intensity spectrum is then Fourier transformed into the time domain to reconstruct the depth-resolved sample optical structure.

The principle of FD-OCT arises from equation (12). Since the reference mirror is now static, $\Delta z = 0$ and maintaining the assumption of an ideal 50:50 beam-splitter an expression for the detected frequency spectrum is obtained:

$$I(\omega) = \frac{1}{4} S(\omega) \{H(\omega) + 1\}^2.$$  \hspace{1cm} (20)
Equation (20) is useful since it shows that an arbitrary source spectrum can be readily deconvolved from the sample response by dividing the output intensity spectrum by the measured source spectrum; a similar deconvolution technique for TD-OCT has been described previously (Wang 1999). The depth resolved structural data are obtained from the Fourier transform of $I(\omega)$ into the time domain interference pattern $I(t)$:

$$I(t) = FT\{I(\omega)\}$$

(21)

FT denotes the Fourier transform operation. The interference pattern can then be displayed as a function of optical time of flight $t$ or equivalent TD-OCT “reference mirror” displacement $\Delta z$. In a real system, the output intensity spectrum $I(\omega)$ is a set of $N$ discrete data points corresponding to an intensity measurement at each detector in the array. Therefore, the Fourier transform can be achieved by means of a fast Fourier transform (FFT) algorithm on a personal computer or in hardware. The Fourier transformed result is composed of a series of $N/2$ discrete steps in time $\Delta \tau$ determined by the detected spectral width $\Delta \Omega$:

$$\Delta \tau = \frac{2\pi}{\Delta \Omega}$$

(22)

The detected spectrum can be approximated by the relation

$$\Delta \Omega = 2\pi c \frac{\Delta \lambda}{\lambda^2}$$

(23)

and substituted into equation (22). The conversion into the spatial domain is achieved by multiplying both sides of equation (23) by $c/n_{ave}$, where $n_{ave}$ is an assumed average sample refractive index. Therefore, the maximum depth $Z_{max}$ is determined by multiplying equation (23) by the number of time domain points $N$ and dividing by 2 to take into account the double pass of the light through the sample:
\[ Z_{\text{max}} = \frac{1}{4n_{\text{ave}} \Delta \lambda} N. \] (24)

Hence, if a detector array consists of \( N = 1024 \) elements, and the source has center wavelength \( \lambda_0 \approx 800 \text{ nm} \) and bandwidth \( \Delta \lambda = 50 \text{ nm} \), then we find that the maximum axial scan depth for a sample with average refractive index \( n_{\text{ave}} = 1.3 \) must be \( Z_{\text{max}} = 2.5 \text{ mm} \). Equation (24) shows that the maximum axial scanning depth scales linearly with the number of detector elements.

### 2.2 Light scattering spectroscopy (LSS) for nuclear morphology measurements

90\% of all human cancers, including breast, lung, prostate, colon, pancreatic and skin cancers, arise in the epithelial tissue that lines body surfaces but none of these cancers become highly dangerous to a person unless they invade through the underlying basement membrane and begin to spread to other tissue (Conger 2009). Although these are readily treatable provided they are diagnosed in one of the preinvasive stages, early lesions are often almost impossible to detect (Backman et al. 2000).

Before they become invasive, at stages known as dysplasia and carcinoma in situ, early cancer cells alter the epithelial-cell architecture. In particular, the nuclei become enlarged and crowded. While the diameter of non-dysplastic cell nuclei is typically 5–10 \( \mu \text{m} \), dysplastic nuclei can be as large as 20 \( \mu \text{m} \) across. Moreover, they become hyperchromatic (that is, they stain abnormally darkly with a contrast dye as a result of changes in their chromatin content). The current gold standard for detecting cancer of epithelial tissues is the histopathologic analysis of biopsy samples. Biopsy samples are excised from the tissue under examination and then fixed, sectioned, stained, and ultimately examined by a pathologist for morphological abnormalities. Although this procedure is the standard practice for cancer diagnosis, there are several drawbacks to this approach, including the subjectivity of diagnoses, the inherent invasiveness of biopsies, the time delay between biopsy and diagnosis, and the poor coverage of at-risk tissue.
It is clear that improved screening and diagnostic technologies are needed to overcome these limitations. In recent years, large amounts of research have focused on developing optical spectroscopic and imaging techniques for early cancer detection (Panjehpour et al. 1996; Wallace et al. 2000; Pfau et al. 2003; Kendall et al. 2003) because such techniques hold great promise to overcome the limitations of the traditional biopsy listed earlier. The three main spectroscopy techniques used in biomedicine are fluorescence spectroscopy, Raman spectroscopy and light scattering (reflectance) spectroscopy (LSS) (Tunnell et al. 2003; Wong Kee Song & Wilson 2005). While the two former techniques are used to gain biochemical information, LSS can give information about the structure and morphology of the tissue.

In the last two decades much work has been carried out in measuring the size of epithelial cell nuclei using LSS to detect cancer cells in vivo and in vitro, as well as to characterize particle size using artificial microspheres and cellular monolayers (Mourant et al. 1995; Perelman et al. 1998; Backman et al. 2000; Boustany et al. 2001). Central to this work is the idea that the cellular organelles of epithelial tissue can be considered as spheroidal scatterers whose interactions with light are governed by Mie theory (Hulst & Hulst 1957; Wang & van de Hulst 1991; Stübinger et al. 2008). Mie scattering theory provides a closed-form description of optical scattering from spheroidal particles as a function of particle size, refractive index, wavelength, observation angle, and optical polarization. Mie theory describes the scattering process in closed form for simple systems of spherical particles under coherent illumination. For single-particle systems, the frequency of the modulation on the backscattered spectrum is proportional to the particle size. The modulation characteristics of the backscattered spectrum are also related to the density of the scattering particles. Therefore, by examining the spectral modulations present in LSS signals, variations in the size and density of scattering particles in biological tissue samples may be differentiated. Since the cells that compose various types of tissue typically have varying organelle sizes and densities, examining spectral modulation may lead to contrast enhancement of different cell types. Furthermore, changes in cell size, nuclear size and mitochondrial density caused by cancer may also be indirectly visible using light scattering microscopy.
Recently, LSS has been implemented with low-coherence interferometry (LCI), which offers the possibility of depth-resolved analysis of the LSS signal (Yang et al. 2000; Wax et al. 2003; Pyhtila et al. 2003; Pyhtila & Wax 2004). However, there remain three important limiting factors in the LSS studies to date. First, because the LSS typically utilizes collimated beams or focusing lenses with very low numerical aperture (NA), there usually is very poor lateral resolution. Second, the collected back-scattered signal intensity in LSS is low because the collection efficiency is proportional to the NA of the focusing lens. Third, the penetration depth of LSS is quite limited due to the effect of multiple-scattering (for non-LCI-based LSS). Because of these shortcomings, LSS, to date, has primarily been used as a functional analysis method rather than a functional imaging method.

2.3 Spectroscopic OCT (SOCT) for nuclear morphology measurements

While LSS has the limitations mentioned above preventing it to be used as a functional imaging method, OCT system showed to be a very good candidate for spectroscopic depth-resolved analysis. This is due mainly to its high NA lens allowing it to achieve high lateral resolutions between 1-10 μm (Huang et al. 1991; Fercher et al. 2003). Moreover, since OCT utilizes heterodyne detection and broadband coherence gating, OCT signal is mostly due to single scattering events within the coherence gate. Therefore, current state-of-the-art OCT systems have deep penetration (1-3 mm in typical tissue) and 1-3 μm depth resolution. It was not long after the development of OCT that the first spectroscopic OCT (SOCT) imaging was described (Kulkarni & Izatt 1996) and, subsequently, demonstrated endogenous contrast in tissue imaging (Morgner et al. 2000; Leitgeb et al. 2000).

Spectroscopic Optical Coherence Tomography (SOCT) extracts depth-resolved spectra that are inherently available from OCT signals. The back scattered spectra contain useful functional information regarding the sample, since the light is altered by wavelength dependent absorption and scattering caused by chromophores and structures of the sample (Robles et al. 2011; Yi et al. 2013).
As illustrated in Figure 2.8, signal processing for SOCT splits up into four separate blocks: OCT data processing, spectral analysis, the calculation of a spectroscopic metric and the color map (‘staining’) (Jaedicke et al. 2013).

As illustrated in Figure 2.8, signal processing for SOCT splits up into four separate blocks: OCT data processing, spectral analysis, the calculation of a spectroscopic metric and the color map (‘staining’). However, the two aspects that dominate the performance of SOCT are the spectral analysis processing method used to obtain the spatially-resolved spectroscopic information and the spectroscopic metrics used to visualize and interpret relevant sample features (Jaedicke et al. 2013).

OCT processing

If the OCT data are obtained by a FD-OCT system, the first task that is done is re-sampling the raw interferometric spectra from the wavelength to the wavenumber domain. Subsequently, if an intensity based standard OCT processing is going to follow which is most of the times the case, the spectra are Fourier transformed to yield the backscattering amplitude as a function of depth.

Spectral analysis

The choice of the spectral analysis is an important consideration in SOCT since different methods have a strong impact on the results (Xu, Kamalabadi, et al. 2005; Graf & Wax 2007; Kartakoullis et al. 2010). Therefore, a brief description of the available methods is given in this section.
In order to prevent confusions it is noted that although in section 2.1.3 the intensity $I(\omega, \Delta z)$ of an OCT system was expressed as a function of the frequency $\omega$ and the “reference mirror” displacement $\Delta z$, in the following analysis intensity is expressed in wavenumber $k$ since this is a more common notation in the OCT literature, and the “reference mirror” displacement $\Delta z$ is represented by the symbol $z$ as it is more convenient when expressing equations in spatial ($z$-) domain. Adopting the new notation, the intensity of an OCT system can be expressed as:

$$I(k, z) = T_r T_s S(k) |H(k)|^2 + T_r T_s S(k) + 2T_r T_s \Re\{S(k) H(k) e^{-i2n_{air}kz}\}, \quad (25)$$

with wavenumber $k = 2\pi/\lambda$ and optical path length difference $2n_{air}z$, such that $z(n_{air}/n)$ is the assigned depth location in the tissue with refractive index $n$. For simplicity, we assume $n = n_{air} = 1$ in the following analysis. We start with OCT data in spatial ($z$-) domain, either directly acquired (time-domain, TD-OCT) or the result of signal processing after Fourier-domain (FD-OCT) detection, and we assume real-valued representation of the OCT interferograms (i.e. including the modulation term). Together, the wavenumber $k$ and optical path length difference $2z$ form the fundamental Fourier pair in OCT data analysis:

$$I(2z) \xrightarrow{\mathcal{F}} I(k) \quad (26)$$

where $\mathcal{F}$ denotes the Fourier transform operation. Since the wave number $k$ is directly related to wavelength $\lambda$, wavelength dependent spectra $S(\lambda)$ can be obtained from the backscattered OCT signal.

The goal of SOCT is to obtain information in the $\lambda$– and $z$–domain simultaneously, with high resolution in both $\lambda$ and $z$. Due to wavelength-dependent scattering and absorption by the different structures in tissue, the spectral content of the OCT signal changes with depth. If we would directly apply the Fourier transform on the OCT signal (Eq. (2)), either the depth, or wavelength varying information will be lost – depending on time, or spectral domain signal acquisition, respectively. Instead, depth-resolved spectroscopic information can be obtained
using time-frequency (TF) analysis methods (or, for the present context: spectral/spatial analysis). The four accepted methods for spectral-spatial analysis in SOCT are the following (Bosschaart et al. 2013).

1. *Short time Fourier transform (STFT)*

The short-time Fourier transform (STFT) is the most widely applied spectral/spatial analysis method:

\[
STFT(k, z; w) = \int_{-\infty}^{+\infty} l(z') \cdot w(z - z'; \Delta z) \cdot e^{-ikz'} \cdot dz',
\]

(27)

where \(w(z, \Delta z)\) is an analysis window confined in space around \(z\) with spatial width \(\Delta z\), for example a Gaussian function (Xu et al. 2004; Bosschaart et al. 2009; Yi & Backman 2012; Fleming et al. 2013). The multiplication with a relatively short window effectively suppresses the signal outside the analysis point \(z \pm \Delta z/2\). Physically, the STFT can be considered as the result of passing a signal through an array of band-pass filters with linearly increasing center frequency and constant bandwidth which is inversely proportional to \(\Delta z\). Thus, there is an inherent trade-off between spectral and spatial resolution. A window with short spatial width \(\Delta z\) will localize the signal well in space but will have reduced \(k\)-resolution; conversely a signal with long spatial width will be less well localized in space with the benefit of increased spectral resolution. For a Gaussian window, a spatial domain width \(\Delta z\) will yield a spectral (wavenumber) resolution of \(\Delta k = 1/(2\Delta z)\), or equivalent wavelength resolution \(\Delta \lambda\):

\[
\Delta \lambda = \frac{\lambda^2}{2\Delta z}.
\]

(28)

In practice, starting with a real-valued time-domain A-scan, a spatial window \(w_z\) with center at \(z\) and width \(\Delta z\) is chosen. This window is multiplied with the A-scan and subsequently Fourier transformed with \(z \rightarrow k\) (Equation (27)). Repeating for every \(z\) results in a complex valued \(STFT(k, z; w_z)\) spectrogram with \(z\)-axis equal to that of the time-domain A-scan, and
k-axis from $k = 0$ to $k = \pi/2\delta\zeta$ where $\delta\zeta$ is the sampling increment of the A-scan. The resolution in the spatial domain is given by the window width $\Delta z$; the resolution in the spectral domain is $\Delta k = 1/2\Delta z$ assuming a Gaussian window (Equation (28)). When operating on real-valued Fourier domain interference spectra, a $k$-domain window $w_z$ centered at $k$ and width $\Delta k$ is chosen, and multiplied with the interference spectrum. Fourier transform $k \rightarrow z$ at each $k$ then yields the complex spectrogram $STFT(k, z; w_z)$. The spectral range equals that of the interference spectrum; the spatial range runs from $z = 0$ to $z = \pi/2\delta k$ where $\delta k$ is the spectral domain sampling interval. For further processing the amplitude of the complex spectrogram is taken.

2. Wavelet transforms

The wavelet transform was introduced to partially overcome the trade-off in spectral/spatial resolution of the STFT, by adjusting the window size to the frequency being considered. However, in the practice of SOCT, wavelet transforms have only been applied using Gaussian windows with fixed bandwidth (Morgner et al. 2000), which is essentially the same as the STFT (Bosschaart et al. 2013).

3. Wigner-Ville distribution (WV)

Bi-linear spectral/spatial distributions (e.g. Cohen’s class TFTs) do not suffer from the resolution trade-off between both domains. The most important member of this class is the Wigner-Ville distribution (Leitgeb et al. 2000):

$$WV(k, z) = \int_{-\infty}^{+\infty} I(z + z')I^*(z - z') \cdot e^{-ikz'} \cdot dz'.$$

The Wigner-Ville distribution is the Fourier transform of an autocorrelation measure of the signal $I(z)$. The result of Equation (29) is complex; for further processing the absolute value is taken. Although bilinear transforms can better concentrate the time-frequency response, they are also subject to cross-terms, where the signal $I(z)$ effectively interferes with itself, which can be difficult to interpret. These cross-terms can be reduced using low-pass filtering.
methods such as the smoothed-pseudo WV, particularly since OCT signals typically have low fractional bandwidths and, thus, the cross-terms are found outside the signal band (Xu, Kamalabadi, et al. 2005). But then, the STFT compromise between spatial and spectral resolution is replaced by a compromise between the joint spectral/spatial resolution and the level of suppression of the interference terms.

4. Dual window method (DW)

Robles et al. showed the pseudo WV can also be obtained by STFT analysis using two k-domain window sizes $\Delta k_1 \gg \Delta k_2$ (Robles et al. 2009) (or equivalently: two z-domain window sizes $\Delta z_1 \gg \Delta z_2$). The result $STFT_1(k,z) \times STFT_2(k,z)$ is mathematically equivalent to a pseudo WV with window widths of $w_k(k,\Delta k = \Delta k_2/2)$ in the spectral domain and window $w_z(z,\Delta z = 1/(2\Delta k_1))$ in the spatial domain. As for the WV, any remaining interference terms may carry information, for example on average spatial scatterer separation. Robles et al state that the DW method is superior to the STFT in terms of the trade-off in spectral/spatial resolution – i.e. the spatial resolution equals the width of the narrow spatial window, and the spectral resolution equals the width of the narrow spectral window. When applied in practice, interference terms between nearby reflectors (both located within one long spatial window) appear as frequency modulations on the recovered spectra. These modulations can be removed by low-pass frequency domain filtering (Robles et al. 2009).

Spectroscopic metric and color map

The calculation of the spectroscopic metric and the generation of the color map are closely related (Jaedicke et al. 2013). Figure 2.9 shows a block diagram of the steps and methods involved in calculating the various spectroscopic metrics and the generation of the color map. There are four blocks: pre-processing, feature reduction, pattern recognition and display.

Pre-processing is necessary in order to reduce the noise and to correct for system specific features. First, the depth-resolved spectra are averaged using a smoothing two dimensional
Gaussian filter (Jaedicke et al. 2013). The edges of the spectra, which are lower in intensity, are more susceptible to noise compared to the center spectral region. Hence, this part of data is excluded from the subsequent analysis. Finally, the depth-resolved spectra are normalized by scaling them from zero to one. This preprocessing reduces speckle-like noise and excludes intensity information from subsequent analyses (thus only taking spectral fluctuations into account).

The aim of the spectroscopic metric is to reduce the dimensionality of the spectra and to highlight the relevant sample properties. Some of the most common methods for feature reduction are listed here:

- **Principal Component Analysis (PCA):** a simple and non-parametric tool for data analysis (Kartakoullis et al. 2010).
- **The SUB metric:** this method is similar to the way the human eye detects colors. The spectrum is divided into three sub-bands using weighting functions and the integrated values of each sub band are directly assigned to red, blue and green hue in the RGB color model (Robles et al. 2011; Xu et al. 2006).

- **Autocorrelation function (ACF) method:** the bandwidth or a combination of different bandwidths about the zero lag of the autocorrelation function (ACF) of the spectra is used as an indicator for the scattering properties of the sample (Adler et al. 2004; Wax et al. 2003; Tay et al. 2012).

- **The COM metric:** The center of mass of each spectrum is calculated reducing the whole spectrum to one single value. Next, this value is used to calculate a color map according to the hue channel in the hue, saturation and value (HSV) color model (Morgner et al. 2000).

- **Phasor Analysis (PHA):** Each spectrum is reduced to two parameters given by the real and imaginary parts of the demodulated (or depth resolved) spectrum’s Fourier transform at a particular frequency (Digman et al. 2008). This method has been shown to be fast and effective for unmixing fluorescence microscopy spectral images (Fereidouni et al. 2012).

In pattern recognition one can distinguish between unsupervised and supervised methods. Unsupervised methods do not require a priori measurements, while supervised methods need a learning phase, where a set of labeled data needs to be available. The accuracy of pattern recognition approaches can considerably be better when the number of input variables is reduced by feature reduction. Thus the feature reduction methods are used in two ways: (1) to reduce the dimensionality of the data in order to display the spectroscopic information directly using a color map and (2) to reduce the number of the features for the subsequent pattern recognition analysis.

One common method of supervised algorithm for pattern recognition is the k-means clustering which assigns the spectra to one of a predefined number of clusters (Bishop 2007). This number of clusters is not known for many applications of SOCT. Thus, another algorithm can be used that determines the number of clusters in a data set. Using k-means
clustering, small Euclidian distances in the feature space, are grouped together and are assigned to similar colors e.g. red and yellow, while clusters with relatively larger distances are assigned to colors with more contrast e.g. red and blue.

Moreover, a commonly used unsupervised learning algorithm is the Self Organizing Map (SOM) which is based on artificial neural networks (Kohonen 1990). SOM can be used in the same way as K-Means for clustering, and then have its spectroscopic information encoded directly into the three channels of the RGB color model (SOMRGB) (Gross & Seibert 1993).

Feature reduction is necessary for the visualization of the multidimensional data as a color map, which is the most intuitive way to display the information content of the spectroscopic analysis. Since the standard intensity analysis, which is performed in OCT, can give complementary information regarding to the spectroscopic analysis, it is useful to combine both, the results from intensity and spectral analysis, in one image. Therefore, the intensity distribution is usually encoded in the intensity of the image, while the spectroscopic metric is encoded in the color of the image. Typically the color map in SOCT is used in two different ways: (1) one can encode continuous features, e.g. the center of mass, directly into color. In this case, the interpretation is done by the user, who is looking for areas in the color map with similar hues. (2) On the other hand, if one is only interested in differentiating a limited set of sample properties, a discrete staining can be used. Therefore, to classify the spectra according to the relevant sample properties, pattern recognition algorithms can be used and the results can be displayed in discrete colors, e.g. blue and red.
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3.1 Spectroscopic metric for scatterer size estimation

Over the last two decades, much research has been done in order to find a spectroscopic metric that can accurately measure the size of epithelial cell nuclei using LSS since such a metric could contribute to the early detection of cancer (Perelman et al. 1998; Gurjar et al. 2001; Xu, Carney, et al. 2005; Kartakoullis et al. 2010; Tay et al. 2012; Seck et al. 2015). Most of the methods proposed until today are based on the assumption that epithelial cell nuclei can be considered as spheroidal scatterers whose interactions with light are described by Mie theory (Sloot et al. 1988; Beuthan et al. 1996). Thus, some of these methods, investigate the possibility to do curve-fitting of the backscattered spectra obtained from the OCT or LCI signal to the theoretical prediction (Wax et al. 2002; Robles & Wax 2010; Seck et al. 2015). The drawback of this process is that it needs an exhaustive search of possible scattering sizes and knowledge of the precise refractive index of the scatterer and the surrounding medium (Robles et al. 2010). Moreover, it has to deal with the fact that spectral shifts may occur in the experimental measurements if the incident beam waist spot size is small, or comparable to the wavelength, since Mie theory does not refer to Gaussian beams but rather to plane waves (Xu, Carney, et al. 2005).

On the other hand, many studies have been done based on the observation derived from Mie theory that as the particle size increases, so does the oscillation “frequency” on the backscattered spectrum. Therefore, by examining the number of oscillatory patterns in the experimental measurements, variations in the size of scattering particles can be detected. Basically, there are two main approaches that exploit this feature. The first approach, proposed by Wax et al (Wax et al. 2003), involves some pre-processing to the backscattered spectrum followed by a Fourier transform, yielding the correlation function as it is denoted. The maximum of this function give the differences in optical path length between dominant scattering features in the analyzed region. In the case of a microsphere phantom, they suggest that the local oscillations predominately result from scattering by the front and back surfaces.
of a single bead, and thus the correlation function maximum indicates the round-trip optical path length through the scatterer. While this method has been followed in many studies working on phantom tissues and biological tissues, and has showed quite accurate estimates on the scatterer sizes (Wax et al. 2003; Graf & Wax 2005; Robles et al. 2009; Graf et al. 2009; Robles & Wax 2010; Robles et al. 2010), when the light source spectrum is limited and does not allow a sufficient number of oscillations to be captured in the backscattered spectrum, it is hard to distinguish the maximum peak from the low-frequency components (Oldenburg et al. 2007).

The second approach for estimation of scatterer size, proposed by Adler et al., involves the autocorrelation width of the backscattered spectrum (Adler et al. 2004). As they state, backscattered spectra with high spectral modulation produce autocorrelation functions that fall of rapidly away from the central point, while spectra with low spectral modulation produce autocorrelation functions that fall of slowly. In their work, they demonstrated the potential of this method on OCT images of a developing zebrafish embryo by encoding the bandwidth of the autocorrelation function at the 90% of the peak value in the hue of a HSV color map, while the intensity of the backscattered signal was encoded into saturation and value. In this way, they achieved improvement to the contrast enhancement of the OCT image. The strong point of this spectroscopic analysis technique is that it does not depend on the distribution of optical power over absolute wavelength and thus it is insensitive to major sources of spectroscopic noise.

In the next few years, variations of this technique were adopted in other studies. For example, Oldenburg et al. used the autocorrelation width of backscattered spectra at 80% of the peak autocorrelation value in order to enhance the contrast of OCT images of macrophages and fibroblasts (Oldenburg et al. 2007), whereas kartakoullis et al. and Jaedicke et al. combined the autocorrelation function with PCA analysis and clustering algorithms with the intention of differentiating phantom samples consisting of microspheres with different diameters (Kartakoullis et al. 2010; Jaedicke et al. 2013). Additionally, Tay et al. suggested that the use of multiple bandwidths can improve the sensitivity of this method to scatterer size
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demonstrating it in spectroscopic OCT images where solutions of 0.5 and 45 μm microspheres could clearly be distinguished.

In this study, we examine the possibility of “measuring” the degree of spectral modulation in backscattered spectra obtained by OCT images of microscale particles and comparing it with predictions from Mie theory in order to make estimations of particle sizes. All the Mie spectra that are used in this study were calculated based on the Fortran BHMIE (Bohren & Huffman 1998) which has been modified and converted to Matlab code. Figure 3.1 shows

![Graphs showing backscattered Mie Spectra for different diameters](image)

**Figure 3.1**: Backscattered Mie Spectra for (a) 10.0 μm, (b) 10.5 μm and (c) 11.0 μm scatterers over the wavelength range from 700 to 1400 nm with medium and sphere refractive indices set at 1.47 and 1.59 respectively. As we look to larger (smaller) scatterers the shape of the curve shifts to longer (shorter) wavelengths.
the Mie spectra for the 10.0, 10.5 and 11.0 μm scatterers in the wavelength range between 700 and 1400 nm. The medium and sphere refractive indices were set at 1.47 and 1.59 which are typical values for this kind of experiments. Looking at these spectra, we see that there are high-frequency oscillations that speed down as we go from the shortest to the longest wavelength. Something that we were expecting and confirmed with the calculations of these curves is that a pattern we see on a spectrum for some particle at some wavelength range is the same with the one we see for a larger particle at higher wavelengths. However, when looking at the three spectra we realize that even the 0.5 μm difference in diameter between the scatterers is large enough to make a noticeable shift of the shape of the curve to the longer wavelengths. Therefore, we see that despite the complexity of Mie scattering calculations, the curves we get at the examined range demonstrate strong features that we can use for our benefit. Last, when looking at the spectra we also notice low-frequency oscillations but this feature has limited potential for use because of the relatively narrow spectral range of light sources used in OCT systems.

After making our observations in the wavelength range between 700 and 1400 nm, we focused our attention at the range between 1230 and 1390 nm which is the range where the light source of the OCT system we use in our experiments operates. However, because the OCT analysis that follows later is done in the wavenumber (k-) domain and not at the wavelength (λ-) domain, all the spectra that are shown in the rest of this work are in the k-domain. Figure 3.2 illustrates the backscattered Mie spectra for the case of 6.0, 10.0 and 16.0 μm scatterers in the wavenumber range we are interested. The selection of the scatterer sizes and the refractive indices of the scatterers and the medium were made according to the specifications of the microsphere samples we examine later in the experiments. As we can see, the number of oscillations in the three spectra is much smaller than before; therefore the high-frequency oscillations do not show to speed up in the direction from the smallest wavenumber to the largest, as we would expect to see in a larger range. Nevertheless, we observe that the larger the size of the scatterer is, the more oscillations are shown in the spectrum, which is in agreement with what was mentioned before in the discussion for the autocorrelation method.
However, in this study, we tried to gain further insight into the relationship between the scatterer size and the degree of modulations in the backscattered spectrum. Therefore, we calculated the autocorrelation of this three Mie spectra and made some observations regarding the position of the peaks and the decay rates (1st column of Figure 3.3). Particularly, we see that the larger the scatterer is, the closer are the peaks to the zero lag. Moreover, when looking at the correlation function of the 6.0 and 10.0 μm scatterers we notice a large decay rate, whereas for the 16.0 μm scatterer, the oscillations seem to be on a negative slope. These
two characteristics on the autocorrelation functions are caused by a low-frequency baseline in the spectrum. While we can still distinguish the peaks on the correlation functions for the theoretical Mie spectra, in the case we analyze backscattered spectra obtained from OCT images, this task becomes much more difficult.

Thus, we extended the autocorrelation method by taking the first derivative of the spectra before calculating the correlation function removing in this way significantly the baseline effects. Doing so, we eliminate differences in the peak intensities in a spectrum and therefore placing more emphasis on the peak positions. The robustness of this extended version is
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demonstrated in Figure 3.3 (2nd column) where the correlation functions decay much more slowly.

Subsequently, we plotted a graph with the bandwidth of the correlation functions that are based on the spectrum derivative, as a function of scatterer size assuming a light source in the range between 1230 and 1390 nm (Figure 3.4). The bandwidth values, or COD bandwidths as we call them for short, are taken at the first minimum peak (marked with red circle in Figure 3.3). The Mie spectra we are interested can be considered as quasi-repeating signals, thus the maximum peaks in their correlation functions should correspond to the frequency of repetition. However, because the spectrum range of the light source we use in our experiments has a relatively narrow range, for small scatterer sizes there is only a minimum peak and not a maximum peak in the correlation function. For this reason we choose the first minimum peak as a metric which corresponds to the half of the frequency of repetition.

![Figure 3.4](image)

*Figure 3.4:* Autocorrelation bandwidth at the first minimum peak (or so-called COD bandwidth) plotted as a function of scatterer size. The blue line corresponds to the theoretical curve and the green line to the 4th order approximation curve. We assume the spectral range of the light source is in the range between 1230 and 1390 nm, and the medium and scatterer refractive indices are 1.47 and 1.59 respectively.
Looking at Figure 3.4, we see that for scatterers with diameters in the range between 5 and 20 μm there is almost a linear relationship between the COD bandwidth and the scatterer size while for scatterers smaller than 5 μm there is a high degree of fluctuation. These fluctuations, shown in the theoretical curve, come from the fact that the corresponding Mie spectra have less than two cycles and thus cannot yield consistent results.

It should be noted that the relation between the COD bandwidth and the scatterer size depends on the spectral range of the light source and the refractive indices of the medium and scatterer. This dependence is illustrated in the next figures where we examine some scenarios. For ease of visualization we show only the approximation curves. In Figure 3.5, the COD bandwidth is plotted as a function of scatterer size for three different medium refractive indices assuming a constant spectral range light source and scatterer refractive index of 1.59. In both three cases the curve is monotonic; however, we observe that in the case with the refractive index of the medium at 1.30 (blue line) the relation of the COD bandwidth and the scatterer size is stronger. This suggests that the difference between the refractive indices of the medium and

Figure 3.5: COD bandwidth plotted as a function of scatterer size for three different refractive indices of the medium (Only the approximation curves are shown). We assume the spectral range of the light source is in the range between 1230 and 1390 nm, and the scatterer refractive index is 1.59.
the scatterer, apart from the backscattering intensity, they may play some role to the degree of modulation in the spectrum.

In Figure 3.6, the size dependence of COD bandwidth is illustrated for different light source bandwidths assuming a constant center wavelength at 1310 nm. We observe that the larger the bandwidth is, the less the fluctuations are present in the theoretical curve for the smaller scatterers (not shown in the figure) which can be explained by the fact that with a large bandwidth more oscillations in the spectrum are seen and thus more consistent results are obtained. Moreover, for scatterers in the range 1-9 μm, a larger bandwidth has a stronger relation between COD bandwidth and size. Nevertheless, for larger scatterers, the 220 nm source bandwidth (blue line) does not show a dependence on scatterer size, whereas the 100 nm source bandwidth (red line) shows an almost linear dependence on scatterer size. The reason that the COD bandwidth for a larger bandwidth stays almost constant for different scatterer sizes is that the shift of the curve’s shape in the spectrum as the size gets larger is relatively small compared to the bandwidth and thus there is not much change in the autocorrelation function. However, while the smallest source bandwidth shows to be more robust, when applied to experimental signals it may not be the best choice since a wide spectral range is required to compensate for different types of noise.

Similar conclusions can be drawn when looking at Figure 3.7 which refers to the case of an 800 nm center wavelength. Moreover, we can see that for scatterer sizes in the range 9-17 μm none of the source bandwidth seems to be a good choice. The 100 and 200 nm bandwidth illustrate a non-monotonic curve in this region while the 40 nm bandwidth would probably be vulnerable to noise. Nevertheless, for scatterers with diameters between 1 and 9 μm, all the three source bandwidth demonstrate a strong size dependence for the COD bandwidth. Additionally, in this region, the fluctuations are much less than in the case with the 1310 nm center wavelength (not shown in the figure). Therefore, in order to use the COD bandwidth as a spectroscopic metric to estimate the diameter of a particle, a knowledge of the medium and particle refractive indices is required as also the use of a light source operating in a suitable spectral range.
Figure 3.6: COD bandwidth as a function of scatterer size for three different wavelength bandwidths of the light source assuming a constant center wavelength at 1310 nm (Only the approximation curves are shown).

Figure 3.7: COD bandwidth as a function of scatterer size for three different wavelength bandwidths of the light source assuming a constant center wavelength at 800 nm (Only the approximation curves are shown).
3.2 OCT system and microsphere samples

To evaluate the feasibility of the COD bandwidth to be used a spectroscopic metric of the scatterer size, we created phantom samples containing polystyrene microspheres and applied the spectroscopic metric on their OCT images. Specifically, two series of samples were created consisted of Polybead® microspheres (Polysciences, Inc., Warrington, Pennsylvania) with 6, 10 and 16 μm nominal diameters, embedded in an acrylamide gel. In the first series of samples the concentration was at such level as to achieve a 300 μm average distance between microspheres (~17 × 10⁻³ particles per imaging volume), whereas in the second series the concentration was about eight times larger.

For the production of the microsphere samples we followed the recipe that was used in a similar study (Kartakoullis 2008). For each microsphere sample, we combined 250 μL of acrylamide (30%) and 20 μL of APS (10%) with a 750 μL mixture of microspheres and dH₂O, in which mixture the microsphere concentration was determined by the desired final concentration in the sample. Subsequently, we used a vortex mixer to better mix the microspheres in the solution and finally we added 20 μL of TEMED for polymerization to occur. After 2-3 minutes the gel was polymerized and ready for imaging.

The refractive index of the matrix was also calculated in Kartakoullis’s work and it was found 1.47. Moreover, the refractive index of the Polybead microspheres, according to manufacturer, is 1.59 which means that the relative refractive index (1.08) approximates biological conditions (the relative refractive index of subcellular organelles in cytoplasm is in the range between 1.03 and 1.10 at visible wavelengths) (Beuthan et al. 1996).

The OCT data for the microsphere samples were obtained using the Santec IVS-300 Swept-Source OCT system (Santec Corp., Komaki, Japan). The system has a center wavelength near 1310 nm with a bandwidth of 160 nm, providing in-air axial and lateral resolutions of 12 and 22 μm respectively (FWHM) and an imaging depth range of 4 mm (in air). The incident power on the samples is over 1 mW while the system sensitivity is about 100 dB.
In Figure 3.8(a), we can see an image of the 6 μm microsphere sample along with a granular “noise”, so-called speckle. Speckle is a common phenomenon from which OCT images suffer and is due to multiple scattering within the probing coherence volume. These scattered signals add coherently; that is, they add constructively and destructively depending on their relative phases, which leads to the speckle noise shown as bright and dark dots in the image. In order to reduce speckles, we adjusted the system to acquire the images by using 256-frame averaging. As it can be seen from Figure 3.8, frame averaging reduces significantly the speckle noise.

For each microsphere sample we took two OCT images; one with the standard method and another one with lateral oversampling of the image as it has been shown to improve the lateral resolution of the OCT system (Boui & Pitris 2012). Both methods produced raw data of 500 A-scans with 2048 samples/A-scan but for the standard method the 500 A-scans were across a 5 mm width whereas for the oversampling method the 500 A-scans were across a 1 mm width. The depth range in both methods is estimated to be around 6 mm. After taking the images from the three samples, we repeated the procedure of imaging focusing on another

*Figure 3.8:* (a) Single vs (b) 256-frame averaged imaging. Frame averaging clearly reduces the speckle noise.
region in each sample, so we can determine the repeatability of our method in estimating scatterer sizes.

### 3.3 Signal processing

The analysis of the raw data acquired from the SS-OCT system was achieved using Matlab R2010a. In the next sections, a brief description of the six matlab codes written for the analysis is presented (the source code is provided in Appendix). Some external functions used in these codes were downloaded from the MATLAB Central File Exchange while the file `getMie_mub.m` was provided by Prof. Costas Pitris. `getMie_mub.m` is the function that takes as input the medium and scatterer refractive indices, a wavelength value and the scatterer diameter, and returns the respective Mie backscattering coefficient.

#### 3.3.1 Create_COD_bandwidth_curve.m

This .m file takes as input parameters the wavelength range of the light source used by the OCT system as also the matrix and microsphere refractive indices in order to construct the graph of COD bandwidth (described in Chapter 3.1) as a function of scatterer size. The values of this graph are saved in the file `CodBw_vs_Diameter.mat` so as to be available for use from other codes.

#### 3.3.2 Load_raw_data.m

The file `Load_raw_data.m` keeps a record of all the raw data used in the experiments, as also some parameters for the analysis. Some of the required fields needed for each file are the followings:

- `f`: denotes the fileID of the raw data file. `fileID` is a unique value for each raw data file that we set so we can easily call them in the codes.
- `filename`: is the name of the raw data file.
- `OverS`: determines if the image was obtained with the oversampling or with the standard method.
• *AreaZ1*: determines the number of rows in the top of an image that correspond to the gap between the sample and the probe. The user has to set this parameter manually after displaying the image with the file *Display_spectrum.m* so that later, in the analysis, this area will be skipped.

### 3.3.3 *Load_TD_signal.m*

The file *Load_TD_signal.m* is a function that takes the *fileID* parameter and returns the complex time-domain OCT signal and its magnitude for the image that the *fileID* corresponds to. Typically, the raw data files obtained from a FD-OCT are in the wavelength domain and need to be re-sampled to the wavenumber domain. However, the OCT system we use in this study does the scaling automatically so we do not have to do manually this step. To get the time-domain signal the spectra are first multiplied by a hamming window function and then Fourier transformed.

Moreover, the magnitude of the time-domain signal that is returned by the file *Load_TD_signal.m* is converted in a logarithmic scale for visual purposes; this is a common tactic in OCT imaging where typically a signal has a dynamic range.

In order to enhance the contrast of the OCT image the function *imadjust.m* is used. Additionally, the function *imopen.m* is called which removes all elements in the image with diameter smaller than a predetermined value, yielding a clearer image.

### 3.3.4 *Display_spectrum.m*

Although not necessary for the creation of the spectroscopic images, the file *Display_spectrum.m* is very useful as it allows the user to experiment with different parameters and decide what values to use for the subsequent analysis. Specifically, in this code, the user sets the position and the size of a window in the spatial-domain, as also some parameters regarding the processing of the spectrum and the code returns the graph of the
processed spectrum with an estimation of the diameter according to the relation between COD bandwidth and scatterer size.

For the processing of the spectrum the following steps are done:

- First, the short time Fourier transform (STFT) that was described in Chapter 2.3 is implemented using a Gaussian window function.
- Subsequently, the depth-resolved spectra are divided by the source spectrum so as to obtain the backscattering profile of the sample at their corresponding spatial regions. The source spectrum was measured by placing a mirror in the sample arm at three different z positions. For each position the output intensity spectrum was derived and the average of these three spectra (Figure 3.9(c)) is used in this code to deconvolve the source spectrum from the depth-resolved spectrum. Figure 3.9(a) shows the OCT

![Image](https://via.placeholder.com/150)

**Figure 3.9:** Deconvolution of the source spectrum from an output intensity spectrum. (a) OCT image of the 16 μm microsphere sample focused on a random microsphere that is examined. (b) Depth-resolved spectrum corresponding to the region marked in (a). (c) Spectrum of the light source, and (d) ratio of the depth-resolved spectrum and the source spectrum corresponding to the backscattering spectrum of the sample in the examined spatial region.
image of the 16 μm microsphere sample with two rectangles in the region that is analyzed. The long green rectangle shows the extent of the Gaussian window used in the spatial/spectral analysis while the shorter red rectangle denotes the Gaussian RMS width (known also as standard deviation) of this window (this style of representation is kept for the rest of this work). Figure 3.9(b) illustrates the depth-resolved spectrum derived from the spatial region inside the window, whereas Figure 3.9(d) illustrates the depth-resolved spectrum after been deconvoluted by the source spectrum. By

Figure 3.10: Depth-resolved spectra of a 16 μm microsphere before and after filtering. (a) & (d) OCT images of the 16 μm microsphere sample with a window (1000 and 300 pixels in axial direction) overlaying the localized region that is analyzed; (b) & (e) depth-resolved spectra before filtering corresponding to the two different window sizes, and (c) & (f) depth-resolved spectra after filtering.
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Doing this it is clear that the peaks on the specific spectrum are at about the same height.

- In the next step, two filters are applied to the spectra. The first one is a median filter that suppresses isolated out-of-range noise while the second one is a low-pass filter (LPF). The order for the median filter and the cut off frequency for the LPF filter are set by the parameters `filterPar1` and `filterPar2` respectively. Moreover, the edges of the spectra are removed since the edges of the source spectrum are lower in intensity.

**Figure 3.11:** Depth-resolved spectra of a 10 μm microsphere before and after filtering. (a) & (d) OCT images of the 10 μm microsphere sample with a window (500 and 300 pixels in axial direction) overlaying the localized region that is analyzed; (b) & (e) depth-resolved spectra before filtering corresponding to the two different window sizes, and (c) & (f) depth-resolved spectra after filtering.
compared to the center spectral region and therefore the edges of the depth-resolved spectra are more susceptible to noise than the central part. Figure 3.10 demonstrates the feasibility of the filters to remove noise induced by the “presence” of a second microsphere in the extent of the window. It is clear to see that when the axial window size is 1000 pixels the presence of the upper microsphere induce high-frequency noise on the spectrum (Figure 3.10(b)) resulting in an overestimation of the microsphere’s diameter, whereas a shorter window of 300 pixels yields a smoother spectrum (Figure 3.10(e)) with correct estimation of the diameter (16 μm). However, when the filters are applied to the spectrum and the edges are removed, the waveforms of the two spectra are similar and both give estimated diameter just below the nominal diameter. Another example is illustrated in Figure 3.11 for the case of a 10 μm microsphere with axial window sizes of 300 and 500 pixels. As before, the original spectrum of the longer window has noise induced by the “presence” of a nearby microsphere and thus leading to an overestimation of the diameter while in the case of the filtered spectra, the two windows give almost the same waveform with estimation of the diameter just below the nominal diameter (10 μm).

Using the code Display_spectr um.m we examined some cases with individual microspheres in the images to understand how the spectrum changes with minor displacements of the spatial window from the center of the scatterer. Figure 3.12 shows OCT images of the 16 μm sample suffered from ghost artifacts which probably are caused by the existence of point-spread function (PSF) side-lobes. We believe that a secondary mode might be present in the laser cavity which introduces side-lobes in the PSF due to the interference of the secondary mode with itself and with the primary mode (Bonesi et al. 2014). Moreover, the high reflectance of the 16 μm scatterers leads the imaging system to saturation of the PSF main peak and therefore the side-lobes have similar amplitudes with the main peak. This explains the appearance of ghosted artifacts in OCT images of the 16 μm microsphere samples whereas the images of the 6 μm and 10 μm are artifact free.
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We could avoid the saturation if the source output power was accessible to the user or if the system was modifiable so that we could add a fibered attenuator in the sample arm (Beaudette et al. 2015), but since we could not do any of these ways we decided to investigate options for deriving information from the artifact. As shown in Figure 3.10(d-f), when using a 300 pixels spatial window the estimated diameter is very close to the nominal diameter (15.1 μm). Nevertheless, when using a shorter spatial window of 100 pixels (Figure 3.12), the degree of modulation in the spectrum is significantly different if the Gaussian RMS width in the spatial window (indicated with red rectangular in the images) includes the interface between matrix

![Figure 3.12: Depth-resolved spectra of a microsphere with a 16 μm nominal diameter at different axial positions. (a), (c) & (e) OCT images of the examined scatterer suffered from ghosted replicas of the sample along the axial direction. (b), (d) & (f) Backscattered spectra of the scatterer derived from different axial position as shown in their respective images (left column).]
and microsphere or not. Specifically, if the RMS width includes the interface, the obtained spectrum has fluctuations that give an estimated diameter of 15.3 μm (Figure 3.12(e)-(f)), while in the case it does not include it, the spectrum is smooth and almost flat with higher intensities (Figure 3.12(c)-(d)).

Figure 3.13 shows spectra of a 10 μm scatterer corresponding to a spatial window of 500 pixel axial size at different depths. In this case, regardless of the window position in the axial direction, as long as the Gaussian RMS width includes the interface between the scatterer

![Figure 3.13](image-url)
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and the matrix, the spectrum will have the same waveform, which in the specific example leads also to a correct estimation of the diameter (~10 μm). However, when the window includes the entire axial length of the scatterer and the interface at both the upper and lower sides, the signal yields a spectrum of quite higher intensity (Figure 3.13(a)-(b)).

Last, we examined the dependence of a depth-resolved spectrum on minor displacements in the lateral direction. Figure 3.14 illustrates the spectra of a 6 μm scatterer obtained from three different A-scans. For the spectral analysis a spatial window of 500 pixels axial size was

![Figure 3.14](image)

Figure 3.14: Depth-resolved spectra of a microsphere with a 6 μm nominal diameter at different lateral positions. (a), (c) & (e) OCT images of the examined scatterer with the rectangles indicating the spatial windows that are analyzed. (b), (d) & (f) Backscattered spectra of the scatterer derived from different lateral position as shown in their respective images (left column).
used. We see that the maximum intensity spectrum occurs when the spatial window is in the center of the scatterer (Figure 3.14(a)-(b)) and as the window goes to the adjacent columns, namely, 1 A-scan for Figure 3.14(c) and 2 A-scans for the Figure 3.14(e), the intensity falls off and the waveform changes slightly its shape.

### 3.3.5 Calculate_spectra.m

*Calculate_spectra.m* takes as input parameters the size of the spatial window that is used in the spectral analysis as also the *fileID* of the image that we are interested, and scans the whole OCT image to find the locations where the spectra fulfill some criteria. These spectra are then stored in output files and in .avi files to find them more easily.

The way the algorithm works is by calculating in each row the spectrum in each of the 500 columns and finding their maximum peak. Then, from these spectra, the algorithm keeps those ones whose peak value is above a threshold value. Additionally, if some localized spectra are closer than 3 adjacent A-scans only the one with the maximum peak is kept. In that way, if we look at the example in Figure 3.14, even though both the three spectra may exceed the threshold value only the spectrum in Figure 3.14(b) would be kept since it has the largest maximum peak and it is considered to represent better the backscattered profile of the scatterer than the other two spectra. Furthermore, to reduce the computation time of the algorithm, only the first in each 50 adjacent rows is scanned as it was found that it does not affect the final result when the spatial window has at least 100 pixels axial size.

### 3.3.6 Create_spectroscopic_image.m

This .m file is the last of the six codes written for the spectroscopic analysis of the raw data files. As mentioned in Chapter 2.3, in spectroscopic imaging the intensity distribution of the OCT signal is typically encoded in the intensity of the image while the spectroscopic metric is encoded in the color of the image, and in our work, this is implemented in *Create_spectroscopic_image.m* where we use the COD bandwidth as a spectroscopic metric and the Hue/Saturation/Value (HSV) color map to represent the spectroscopic information at
each image pixel. Specifically, the hue parameter encodes the spectroscopic information and the OCT intensity is assigned to the value parameter. The saturation parameter takes the value 1 if the hue parameter is non-zero, and the value 0 otherwise.

After running *Calculate_spectra.m* to extract the spectra of a raw data file in output files, *Create_spectroscopic_image.m* takes these output files and makes estimations of the diameters according to the relation between COD bandwidth and scatterer diameter. Then, for each spectrum found in the image, a color is given to the region (image pixels) that it originates from, in order to associate this region with the estimated diameter. In some cases, the spatial regions of two spectra overlap and in these cases the pixels take as hue parameter the one from the spectrum with the maximum mean value.

Subsequently, we do segmentation of the image to multiple segments since such a process allows us to make quantitative analysis of the new spectroscopic metric we propose in this study. Assuming that each segment corresponds to an individual scatterer we can then associate the hue parameter at the centroid of a segment with the diameter of a corresponding “scatterer”. Finally, having labelled all the “scatterers” in the image and associating them with an estimation of the diameter, we process to the statistical analysis that is presented in the next chapter.
4 Results

In this section, we present some of the spectroscopic OCT images created using different parameters, along with statistics related to the size estimation. As described in Chapter 3.3.6, after the coloring of the OCT images according to the spectroscopic metric, a segmentation of the images follows that can give us the “diameter set” of the image. By that, we mean a data set with information of all the segments identified in an image with their positions and their correlated diameter values.

Having calculated the “diameter set” of each image and each set of parameters, we constructed 1-D and 2-D histograms of the estimated diameters. The second variable on 2-D histograms is the depth in the image. In that way, we can investigate the efficiency of the spectroscopic metric we propose as a function of depth. Regarding the 1-D histograms, the value of the bin with the highest peak in each histogram corresponds to the mode of the “diameter set”. The mode value as also the mean and median values, are summarized in Table 2 and Table 3 for comparison. As a measure of dispersion for the mean, median and mode value we used respectively the standard deviation (SD), the median absolute deviation (MAD) and \( \gamma \), the half of width at half maximum (\( \gamma \equiv HWHM = FWHM/2 \)).

4.1 SOCT images and statistics for microsphere samples A-series

Figure 4.1 shows the spectroscopic images of the microsphere samples from A-series in which the microspheres have about 300 μm average separation distance. The axial size of the spatial windows is set at 300 pixels (~220 μm) and the threshold value at 5 (a.u.). It can be seen that in each image a specific color dominates according to the nominal diameter of the microspheres. In the first row, it is the green color which corresponds to diameters with values around 6 μm, in the second one, the cyan (greenish-blue color) for values around 10 μm, and in the third one, the purple for values around 16 μm. The same conclusions can be made when looking at the 2-D histograms in Figure 4.2. However, this figure illustrates better the size distribution across the depth of the image, except for the 6 μm and 10 μm microsphere
samples obtained with the oversampling method (Figure 4.2(b)&(d)) in which the number of segments is relatively low and does not allow any conclusions to be drawn. Looking at the rest of the images, we observe that in the case of the 6 μm and 10 μm samples, as we go deeper in the sample there is a small portion of segments that yields overestimated diameters. Nonetheless, it does not prevent the extraction of useful information. In Figure 4.3, we notice that the 6 μm sample has the narrowest size distribution whereas the 10 μm sample has the widest.

Table 2 summarizes some statistic related to the size distribution of the samples from series A for three different parameter sets. The first parameter set has a threshold value of 5 (a.u.) and is the set that Figure 4.1-Figure 4.3 correspond to, the second one has a threshold value of 20 (a.u.), and the third one has also a threshold value of 20 combined with a 3 A-scan (10 A-scan for oversampling method) lateral averaging (a spatial window of 300x3(10) is implemented as it is thought to increase the signal-to-noise ratio (SNR)). Between the three measures of central tendency, the mode proves to be most the consistent with a good approximation of the expected values. The median yields also good results with generally a smaller dispersion than the mean.

The change of the value from 5 to 20 (a.u.) shows to improve the results since spectra with low SNR are omitted from the analysis. For example, for the image of the 6 μm microsphere sample using the oversampling method, a threshold value of 5 gives a median diameter of 8.8 ± 3.0 μm while a threshold value of 20 gives a median diameter of 6.0 ± 0.7 μm. Moreover, in most of the cases, the lateral averaging shows to improve the dispersal while in some other cases it has the opposite effect.

If we focus our interest to the mode values, we observe that the analysis for the 6 μm microsphere sample gives correctly a mode diameter of 6 μm with a value of γ at about 0.7 μm. Regarding the 10 μm microsphere sample, the mode diameter is at 9 μm for the standard method and 8 μm for the oversampling method, with values of γ between 0.8 and 1.6 μm, and for the 16 μm microsphere sample, the mode diameter is at 17 μm with values of γ between 1.0 and 1.4 μm.
Table 2: Summary statistics of size estimation for different window sizes and threshold values

<table>
<thead>
<tr>
<th>OCT method</th>
<th>Nominal diameter</th>
<th>Lateral averaging (A-scan)</th>
<th>Threshold value</th>
<th>Estimated diameter (μm)</th>
<th>Mean (±SD)</th>
<th>Median (±MAD)</th>
<th>Mode (±(\gamma))</th>
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<td>5</td>
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<td>7.2 ± 2.0</td>
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</tr>
<tr>
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<td>5</td>
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<td>8.7 ± 1.8</td>
<td>8.8 ± 0.8</td>
<td>8 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>16</td>
<td>3</td>
<td>20</td>
<td>16.4 ± 1.6</td>
<td>16.8 ± 0.8</td>
<td>17 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Oversampling</td>
<td>16</td>
<td>10</td>
<td>20</td>
<td>16.4 ± 1.7</td>
<td>16.7 ± 0.8</td>
<td>17 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1: Spectroscopic images of the microsphere samples from A-series. SOCT images of the 6 (a)-(b), 10 (c)-(d) and 16 μm (e)-(f) microsphere samples using the standard method (left column) and the oversampling method (right column). The axial size of the spatial windows is set at 300 pixels (220 μm) and the threshold value at 5 (a.u.).
Figure 4.2: 2-D histograms of the scatterer estimated diameter as a function of depth for the microsphere samples A-series. (a)-(b), (c)-(d) and (e)-(f) correspond to the 6, 10 and 16 μm microsphere samples, while the left and right columns correspond to the standard and oversampling method respectively.
Figure 4.3: Histograms of the scatterer estimated diameter for microsphere samples A-series. (a)-(b), (c)-(d) and (e)-(f) correspond to the 6, 10 and 16 μm microsphere samples, while the left and right columns correspond to the standard and oversampling method respectively.
4.2 SOCT images and statistics for microsphere samples B-series

Figure 4.4 shows the spectroscopic images of the microsphere samples from B-series in which the concentration of microspheres is about 8 times larger than the concentration in A-series. The threshold value is at 5 (a.u.) and the axial sizes of the spatial windows are 300 pixels (~220 μm) for the left column and 100 pixels (~70 μm) for the right. For each image there is a corresponding 2-D and 1-D histogram of diameter illustrated in Figure 4.5 and Figure 4.6 respectively.

It is clear to see that for the denser samples of B-series the 300 pixels spatial window still works fine for the 10 μm and 16 μm microsphere samples but not for the 6 μm sample. The specific sample (6 μm) is slightly denser than the other two and the 300 pixels window is too long to capture individual microspheres in the image. Therefore, the spectra of the scatterers are strongly influenced by the presence of nearby scatterers leading to erroneous diameter estimations.

On the other hand, a shorter window of 100 pixels axial size yields good results for the 6 and 10 μm sample but not for the third one. As discussed in Chapter 3.3.4, when the spatial window is as short as to fit the Gaussian RMS width in the internal area of a scatterer in the image, excluding the interface between the medium and the scatterer, the resulting spectrum is almost flat with relatively higher intensities. These spectra overshadow the existence of the spectra with the structural information, leading again to erroneous diameter estimations.

Last, instead of the STFT, we tried to implement the dual-window (DW) method where two separate Gaussian windows, a narrow and a wide one, are applied simultaneously to the spatial domain data. The transformed data from each window are then multiplied together to achieve a localized backscattering profile with concurrently high spectral and spatial resolutions (Tay et al. 2012). Theoretically, the spatial resolution of the DW method is determined by the size of the narrow window, but in our case, the spectra obtained by the DW method are found to be influenced by scatterers that are located outside the narrow
window. As a result, even when we applied the DW method, we did not find any pair of windows that could yield good results for both the 6 and 16 μm microsphere samples.

Table 3: Summary statistics of size estimation for different window sizes

<table>
<thead>
<tr>
<th>Nominal diameter</th>
<th>Axial Window (pixels)</th>
<th>Estimated diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (±SD)</td>
</tr>
<tr>
<td>6</td>
<td>300</td>
<td>11.7 ± 3.5</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>6.5 ± 2.2</td>
</tr>
<tr>
<td>10</td>
<td>300</td>
<td>9.5 ± 3.3</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>6.7 ± 2.4</td>
</tr>
<tr>
<td>16</td>
<td>300</td>
<td>16 ± 2.0</td>
</tr>
<tr>
<td>16</td>
<td>100</td>
<td>9.5 ± 4.1</td>
</tr>
</tbody>
</table>
Figure 4.4: Spectroscopic images of the microsphere samples from B-series. SOCT images of the 6 (a)-(b), 10 (c)-(d) and 16 μm (e)-(f) microsphere samples using a spatial window of 300 and 100 pixels axial size (left and right column respectively).
**Figure 4.5:** 2-D histograms of the scatterer estimated diameter as a function of depth for the microsphere samples B-series. (a)-(b), (c)-(d) and (e)-(f) correspond to the 6, 10 and 16 μm microsphere samples, while the left and right columns correspond to a spatial window of 300 and 100 pixels axial size respectively.
**Figure 4.6:** Histograms of the scatterer estimated diameter for the microsphere samples B-series. (a)-(b), (c)-(d) and (e)-(f) correspond to the 6, 10 and 16 μm microsphere samples, while the left and right columns correspond to a spatial window of 300 and 100 pixels axial size respectively.
5 Discussion

In this thesis, we proposed a new metric, called COD bandwidth, for estimating the size of a spherical scatterer in a sample using OCT spectral analysis. The COD bandwidth is determined as the bandwidth at the first minimum of the autocorrelation of the first derivative depth-resolved spectrum. In the case where a spectrum corresponds to the backscattering profile of a spherical scatterer, the COD bandwidth of this spectrum shows to have a strong relation with its diameter. Depending on the light source bandwidth and the refractive indices of the scatterer and its medium, the function of the COD bandwidth versus the scatterer diameter may have a monotonically decreasing behavior which in this case would allow us to determine the scatterer diameter based on its spectrum.

We first demonstrated the dependence of COD bandwidth on scatterer diameter by constructing the analogous curves for different spectral ranges and different values of medium refractive index using Mie-theory calculations. The curves suggest that if this method would be used to determine the scatterer diameters in a medium, a light source operating at a center wavelength in the 800 nm range would perform better for diameters in the range between 1 and 9 μm, whereas a light source operating in the 1310 range would perform better for diameters in the range between 5 and 20 μm.

Subsequently, to illustrate the feasibility of this method, we tested it in two microsphere sample (A- & B-) series which were prepared at different concentration levels. Each series consisted from a 6, 10 and 16 μm microsphere sample. Also, the light source of the OCT system was operating at the 1310 nm range. Regarding the A-series with the dilute microsphere samples, the spectral analysis was conducted using the short time Fourier transform and a Gaussian window function of 220 μm (300 pixels) axial length. After the spectral analysis of each OCT image, a segmentation of the image followed which enabled the correlation of each segment in the image with a diameter estimation. The mode value of the diameter estimations for each image showed a good agreement with the nominal diameters of the corresponding samples. Specifically, for the standard method (no lateral
averaging) and a threshold value of 5 (a.u.), a mode diameter of 6 ± 0.7 μm was found for the 6 μm microsphere sample, 9 ± 1.4 μm for the 10 μm sample, and 17 ± 1.4 μm for the 16 μm sample. To test the repeatability of this method, we conducted the same analysis for OCT images taken from different regions in the samples, and we ended up with the same results. Additionally, after the spectral analysis, we created spectroscopic images of the samples using a HSV color map where the COD bandwidth was encoded to the hue parameter while the OCT intensity was encoded to the value parameter.

With respect to the B-series in which the samples were denser than in the first series, the spectral analysis with the STFT and a 220 μm (300 pixels) Gaussian window yielded similar results with the A series except for the 6 μm microsphere sample which was slightly denser than the other two samples and the spatial window was not short enough to correspond to individual microspheres. On the other hand, a shorter window of 70 μm axial size (100 pixels) showed to overcome this problem and give a correct diameter estimation of 6 ± 1.3 μm, but could not give consistent results for the sample with the 16 μm microspheres. Moreover, we tried to implement the dual-window (DW) method using different pairs of narrow and wide windows, but we still did not find any combination of windows that would give good results for the 6 and 16 μm microsphere samples.

Further investigation showed that in order a spectrum to have the structural information of a spherical scatterer the Gaussian RMS width of its spatial window must include the interface between the medium and the scatterer. If the RMS width of a spatial window extends in the internal area of a scatterer in the image without crossing the interface, the spectrum will be almost flat with high intensities, and therefore will not respond to the scatterer backscattering profile. Spectra like these were found in the case of the 16 μm microsphere sample when a short window was used resulting to erroneous diameter estimations. Therefore, the algorithms we developed have to be modified so as to identify this kind of spectra and exclude them from the subsequent analysis.

Regarding the accuracy of the results, if we neglect the two peculiar cases (the dense 6 μm microsphere sample with a long window and the 16 μm sample with a short window), in all
the other cases the estimated diameters are quite accurate with a maximum divergence of 20%. Also, when repeating the analysis for different areas in the samples, we were getting the same results which suggests that the COD bandwidth – scatterer diameter curve we constructed as a calibration curve needs some corrections. This is something we were expecting since in the calculations we had assumed that the matrix and microsphere refractive indices were independent of wavelength and mostly because the Mie theory that our method is based refers to spherical particles under plane wave illumination, rather than Gaussian beam. However, if this method was implemented in an application like detection of cell nuclear changes, which is the ultimate goal of this study, a pre-calibration stage would be necessary using OCT images of the biological tissues and results of the conventional biopsy.

Despite the accuracy of the mode values for the estimated diameters, there is a wide dispersion in the diameter distributions which is even more intense for the denser samples. Even though we formed an idea of how the spectrum is related to the position and the size of its spatial window, we have not yet found a way to exploit this information. The relatively straight method of using a single spatial window over the scatterer area has already demonstrated its applicability for scatterer size differentiation. Nonetheless, we believe that a clever use of multiple spatial short windows around the scatterer area in the OCT image would yield much more accurate and precise results. If we also manage to recognize patterns of these spectra studying individual scatterers in dilute samples, we could then improve our methods for estimating scatterer sizes in much denser samples.
6 Conclusion

In conclusion, we developed a new spectroscopic metric for OCT analysis, called COD bandwidth, that derives information about the degree of modulations in depth-resolved spectra and estimates the size of scatterers in a sample based on Mie theory predictions. We demonstrated the effectiveness of this metric combined with STFT spectral analysis, in creating spectroscopic images of microsphere samples with clear differentiation between different-sized scatterers as compared to images based on other metrics. Moreover, we investigated the dependence of a spectrum on the relative positions and sizes of a scatterer and a window in the spatial domain. Our future efforts will focus on improving the accuracy and precision of this method by using multiple spatial windows rather than a single one. Additionally, we plan to evaluate the applicability of the proposed metric with ex vivo OCT imaging of the large and small intestines.
References


Stübinger, T. et al., 2008. 100 Years of Mie Scattering Theory : Expanded Size Range by Extreme Precision Calculations. , pp.1–5.


Appendix: Source code

The Appendix contains the matlab codes described in Section 3.3.

I. Create_COD_bandwidth_curve.m

```matlab
% =========================================================================
% Create_COD_bandwidth_curve
% Takes the specifications of the OCT system and the microsphere samples in
% order to create the COD Bandwidth Curve (plot & graph) that is used in
% the next codes.
% =========================================================================
clear; clc; close all;

areMieCurvesSaved = 0;
WspCodBandwidth  = 'WSP/COD_Bandwidth.mat';

% =========================================================================
% Create/Load MieCurves
if (areMieCurvesSaved)
    load(WspCodBandwidth)
else
    % --------------------------------------------------------------------
    % set the parameters
    nPar     = 1.59;
    nMed     = 1.47;
    lamdaMin = 1.23;
    lamdaMax = 1.39;
    nKappa   = 2100;
    diamMin  = 1;
    diamMax  = 20;
    diamStep = 0.1;
    % --------------------------------------------------------------------
    
    kMin     = 2*pi/lamdaMax;
    kMax     = 2*pi/lamdaMin;
    kappa    = linspace(kMin,kMax,nKappa);
    nDiam    = [(diamMax-diamMin)/diamStep]+1;
    diameter = linspace(diamMin,diamMax,nDiam);

    MieCurves = zeros(nDiam,nKappa);
tic
    for d = 1:nDiam;
        minutesElapsed = toc/60,
        perc = d*100/nDiam,
        for k = 1:nKappa;
            MieCurves(d,k) = getMie_mub(2*pi/kappa(k),diameter(d),...
                                      1, nPar,nMed,0);
```

end
\end
save(WspCodBandwidth,'MieCurves','kappa','diameter','nPar','nMed',...
'nKappa','nDiam')
end

% =========================================================================
% =========================================================================
% Create graph and output file of COD Bandwidth vs scatterer diameter
lin    = 1:nDiam;
locMin = zeros(1,nDiam);
for d = lin;
    CorrDer = autocorr(diff(MieCurves(d,:)),nKappa-2);
    [y x]   = findpeaks(-CorrDer,'NPEAKS',1);
    if isempty(x);
        error('No COD bandwidth found! Please check parameters.')
        break
    end
    locMin(d) = x;
end

[p s] = polyfit(diameter(lin),locMin,4);
diamLin = linspace(min(diameter(lin)),max(diameter(lin)),1000);
[CodBw delta] = polyval(p,diamLin,s);

save('CodBw_vs_Diameter.mat','CodBw','diamLin')
plot(diameter(lin),locMin,diamLin,CodBw,'LineWidth',2)
xlabel('Diameter \text{\textmu m}','fontsize',14);
ylabel('COD bandwidth (a.u.)','fontsize',14)
legend('Theoretical curve','Approximation curve')

% =========================================================================
% END m-file Create_COD_bandwidth_curve
% =========================================================================
II. **Load_raw_data.m**

```matlab
% Load_raw_data
% keeps a record of the Raw Data and some parameters for the analysis

latPixRes = [10 2];
depPixRes = 5.970/8;
fmax = 2100;
nKappa = fmax;
nfft = 2^14;
MPD = 60;

remEdgeA = 100;
remEdgeB = 360;
LPF_ON = 1;
filterPar1 = 50;
filterPar2 = 3;

f = 1; % fileID ---> f
filename{f} = 'Raw Data d6a256c1.csv';
OverS(f) = 0;
AreaZl(f) = 900;

f = 2;
filename{f} = 'Raw Data d10a256c1.csv';
OverS(f) = 0;
AreaZl(f) = 700;

f = 3;
filename{f} = 'Raw Data d16a256c1.csv';
OverS(f) = 0;
AreaZl(f) = 700;

...
...

f = 6;
filename{f} = 'Raw Data d16a256c1os.csv';
OverS(f) = 1;
AreaZl(f) = 1300;

...
...

% END Load_raw_data.m
```
III. Load_TD_signal.m

```matlab
function [TD magnTD] = loadTDsignal(fileID)

% =========================================================================
% FUNCTION loadTDsignal
% % Takes the fileID and produces the time-domain OCT signal and its
% % magnitude
% =========================================================================

fprintf('
Reading Raw data ...
');
Load_raw_data
fname = filename{fileID};
fprintf('Processing File: 

', fname);
dirname = sprintf('Raw Data/%s', fname);
FD = csvread(dirname, 9, 0);
[m n] = size(FD);

AverAscan = mean(FD, 2);
AscanSm = smooth(AverAscan, 0.1, 'loess');
FD = FD - (AscanSm * ones(1, n));
FD = FD .* [hamming(m) * ones(1, n)];

TD = fft(FD, nfft);
TD = TD(1193:8192, :);

magnTD = 10*log10(abs(TD));
magnTD = magnTD - min(min(magnTD));
magnTD = magnTD / max(max(magnTD));
magnTD = imadjust(magnTD, [0.58 0.74], []);
magnTD = imopen(magnTD, strel('disk', 1));

% figure; imagesc(magnTD), colormap(gray)

end

% =========================================================================
% END FUNCTION loadTDsignal
% =========================================================================
```
IV. Display_spectrum.m

```matlab
clc;
path(path,'C:\Users\Michael\Documents\MATLAB\functions')
load CodBw_vs_Diameter
load sourceFFT
Load_raw_data

if ~exist('fileLoaded');fileLoaded=0; end

fileID = 1; % choose file according to LoadRawData.m

if ~(fileLoaded==fileID);
    [TD magnTD] = loadTDsignal(fileID);
    fileLoaded = fileID;
end

% set the parameters
figure(11)
subplotLine = 3; % 1-3

DZ  = 500;
DX  = 1;
Xc  = 250;
Zc  = 3050;
yMax = 200;

% figure(11), subplot(3,2,(subplotLine-1)*2+1),
% xlim(gca,[Xc-20 Xc+20]), ylim(gca,[Zc-400 Zc+400])

% PLEASE NOTE! change these values ONLY to test their effect.
% To keep the changes, change their values in loadRawData.m.
% remEdgeA  = 0;
% remEdgeB  = 0;
% LPF_ON   = 0;
% filterPar1 = 50;
% filterPar2 = 3;
```

% ---------------------------
% Display_spectrum
% Takes the specifications of window in time-domain image and displays the
% OCT image and the spectrum that corresponds to the window.
% ---------------------------
Display_spectrum.m

```matlab
subplot(3,2,1+(subplotLine-1)*2);
imagesc(magnTD)
colormap(gray);
ylabel('Depth (pixels)')

X1 = Xc+1-round(DX/2);
X2 = Xc-1+round(DX/2);
Z1 = Zc-1+round(DZ/2);
Z2 = Zc-round(DZ/2);

area = real(TD(Z2:Z1,X1:X2));
[r c] = size(area);

% Calculate and save the FFT spectrum
disp('   Calculating spectrum ...');
area = area.* [(gaussmf(1:r,[r/7 r/2]))*ones([1,c])];
temp = fft(sum(area,2),nfft)/c;
Fxx = abs(temp(1:fmax))./sourceFFT;
if LPF_ON == 1;
    Fxx = medfilt1(Fxx,filterPar1);
    Fxx = FilterFunction(Fxx,filterPar2);
else;
end;
nKappa = length(Fxx);
Fxx = Fxx(remEdgeA+1:nKappa-remEdgeB);

sampleCorr_der = autocorr(diff(Fxx),length(Fxx)-2);
[y x] = findpeaks(-sampleCorr_der,'MINPEAKDISTANCE',MPD,'NPEAKS',1);
[y i] = min(abs(CodBw-x));
diamEst = diamLin(i);

subplot(3,2,(subplotLine-1)*2+1), hold on
DX = c;
DZ = r;
rect_H = rectangle('Position',[X1,Z2,DX,DZ],'EdgeColor','g');
rect_sigma = rectangle('Position',[X1,Zc-DZ/7,DX,2*DZ/7],'EdgeColor','r');

title(sprintf('Dep X Lat: %d x %d (pixels)',r,c))
xlabel(sprintf('Central Coordinates [%d %d]',Xc,Zc))

subplot(3,2,(subplotLine-1)*2+2)
plot(Fxx, 'linewidth',2)
ylabel('Intensity (a.u.)')
ylim([0,yMax])
xlim([0,length(Fxx)])
xlabel(sprintf('Estimated Diameter: %0.3g μm',diamEst))
%
% END m-file Display_spectrum
%=====================================================================
```
V. Calculate_spectra.m

```matlab
% =========================================================================
% Calculate_spectra
% Takes the raw data, finds the depth-resolves spectra that satisfy some
% criteria and saves them to separately output files.
% =========================================================================

close all; clear all; clc;
pwd(path,'C:\Users\Michael\Documents\MATLAB\functions')
load sourceFFT
Load_raw_data

% fileIDs of the images to be processed
Files = [1:6];
\mint{matlab}
winsizeZ = 300;
AverageMethod = 0;
CaptureTraj = 1;
MPD = 3;  % MINPEAKDISTANCE for function FINDPEAKS
MPH = 5E3; % MINPEAKHEIGHT for function FINDPEAKS
\textit{tic}

timeCount=0;
for f=Files;
    timeCount = timeCount+1;
    fname = filename(f);
    Oversampled = OverS(f);
    fprintf('Processing File: %s\n',fname);
    if AverageMethod == 1;
        if Oversampled;   winsizeX = 10;  
    else
        winsizeX = 3;  
    end
    else
        winsizeX = 1;
    end
    if Oversampled;
        nWin = 50;
    else
        nWin = 10;
    end
    winsize       = [winsizeZ winsizeX];
    winsize_um    = round([winsize(1)*depPixRes winsize(2)* ...\}
                           latPixRes(1+Oversampled)]);
    dirname       = sprintf('WSP/MaxPeak/FFT_%dx%d/%s', ...\}
                           winsize,fname(10:length(fname)-4));
    moviedir      = sprintf('WSP/Trajectories/win%d_%dx%d/%s', ...\}
                           winsize,fname(10:length(fname)-4));
    if isempty(dir(dirname));    mkdir(dirname);
    end
    if isempty(dir(moviedir));   mkdir(moviedir);
    end
```
Z1 = AreaZ1(f);
[TD magnTD] = loadTDsignal(f);
im = real(TD(Z1:size(TD,1),:));
[m n] = size(im);
dm = 50;
M = (size(TD,1)-AreaZ1(f))/dm;
N = 500;

% =====================================================================
pcount = 0;
qcount = 0;
for p = 1:dm:M*dm
    pcount = pcount+1;
    complspec = (pcount-1) + ((timeCount-1)*M)+0.001;
    remspec = (M*length(Files))-complspec;
    timeRem = (toc/complspec)*remspec;
    disp('----------------------------------------')
    fprintf('Processed %d of %d layers \n', (pcount-1), M)
    fprintf('Time remaining: %5.1f min\n', timeRem/60);
    fprintf('Time elapsed: %5.1f min\n', toc/60);
    if p <= ceil(winsize(1)/2),
        p1 = 1 ;
    else
        p1 = p-ceil(winsize(1)/2);
    end;
    if p >= m-ceil(winsize(1)/2),
        p2 = m;
    else
        p2 = p-1+ceil(winsize(1)/2);
    end;
    % -------------------------------------------------------------
    % Scan on x-direction to find lateral points with maximum peaks and
    % then save their spectra in files.
    SampleMovie = zeros(fmax,ceil(N/MPD));
yMax = 0;
qucount = 0;
FxxTemp = zeros(fmax,N);
FxxPeaks = zeros(ceil(N/MPD),1);
for q = 1:N
    qcount = qcount+1;
    area = im(p1:p2,q);
    [r c] = size(area);
    area = area.*[(gaussmf(1:r,[r/7 r/2])] ones([1,c])];
    temp = fft(area,nfft);
FxxTemp(1:fmax,qcount) = temp(1:fmax);
FxxPeaks(qcount) = max(abs(temp(1:fmax)));
if (qcount>1 & FxxPeaks(qcount) == FxxPeaks(qcount-1));
    FxxPeaks(qcount) = FxxPeaks(qcount)*1.01;
end
end

[y x] = findpeaks(FxxPeaks,'MINPEAKHEIGHT',MPH, ...
    'MINPEAKDISTANCE',MPD);
qcount = 0;
for i = 1:length(x);
    qcount = qcount+1;
    if (x(i)<=ceil(winsize(2)/2)),
        q1=1;
    else
        q1=x(i)+1-ceil(winsize(2)/2);
    end;
    if (x(i)>=N-ceil(winsize(2)/2)),
        q2=N;
    else
        q2=x(i)-1+ceil(winsize(2)/2);
    end;

area = FxxTemp(:,q1:q2);
c = size(area,2);
Fxx = sum(area,2)/c;
Xc = x(i); Zc=p+Z1;
eval(sprintf('save ''%s/FFT_%dx%dwin_p%dq%d'' Fxx Xc Zc M ',...
    dirname,winsize,pcount,qcount));

if CaptureTraj == 1;  SampleMovie(:,qcount) = abs(Fxx);  end
end

% Capture videos with the spectra.
if CaptureTraj == 1 && ~isempty(x);
    figure(12)
    subplot(1,2,1)
    imagesc(magnTD), colormap(gray);
    hold on,
    y_formatstring = '%3.0f';
    ytick = get(gca, 'ytick');
    for i = 1:length(ytick)
        yticklabel(i) = sprintf(y_formatstring, ytick(i));
    end
    set(gca, 'yticklabel', yticklabel)
clear mov
mov(1:length(x)) = struct('cdata',[],'colormap',[]);
seta('nextplot','replacechildren')
clear rect_H
yMax = max(max(SampleMovie));

for q1 = 1:length(x);
    figure(12)
    subplot(1,2,1)
    hold on
    if (exist('rect_H','var'))
        delete(rect_H)
        clear rect_H
        delete(rect_sigma)
        clear rect_sigma
    end
    z1 = p+Z1;
    x1 = x(q1);
    DZ = winsize(1);
    DX = winsize(2);
    rect_H = rectangle('Position', ... 
    [x1-DX/2,z1-DZ/2,DX,DZ],'EdgeColor','g');
    rect_sigma = rectangle('Position', ... 
    [x1-DX/2,z1-r/7,DX,2*r/7],'EdgeColor','r');
    xlabel(sprintf('Coord: [d d]',x1,z1))
    title(sprintf('Dep X Lat: %dum x %dum',winsize_um))

    subplot(1,2,2)
    Fxx = SampleMovie(:,q1);
    hold on
    plot(abs(Fxx))
    xlim([0,fmax]), ylim([0,yMax])
    xlabel(sprintf('p=%d, q=%d',pcount,q1))
    mov(q1) = getframe(gcf);
end

moviename = sprintf('%s/%s_P%d',moviedir, ... 
            fname(10:length(fname)-4),pcount);
% FFDS reduces sizes but often creates damaged files
movie2avi(mov, moviename, 'compression', 'none');
clear mov
close(figure(12))

shutdown(60) % to cancel: shutdown(-1)
VI. *Create_spectroscopic_image.m*

```matlab
% ==---------------------------------------------------------------
% Create_spectroscopic_image
% ==
% takes the input files with the spectra and creates a spectroscopic image
% along with the statistics for the size distribution.
% ==---------------------------------------------------------------

clear; clc;
path(path,'C:\Users\Michael\Documents\MATLAB\functions')
load CodBw_vs_Diameter
load sourceFFT;
Load_raw_data

% ---------------------------------------------------------------
% set the parameters

Files      = [1];
LoadWSP    = 0;  % 1: the WSP of the file(s) already exists
winZ       = 300;
AverMethod = 0;  % 1 or 0
P       = 1:200;  % choose the rows to process (P=1:200 for all rows)
Q       = 1:100;
MaxHue  = 20;
MeanThr = 20;

% ---------------------------------------------------------------

NP = length(P);
NQ = length(Q);

for f = Files;  % choose filename(sphere)
    XcorrDer = zeros(NP,NQ);
    if length(P) == 1000;  P = 1:Pmax(f); end
    Oversampled = OverS(f);
    if Oversampled;
        DX = 50;
        if AverMethod,  winX = 10;  else winX = 1; end
    else
        DX = 16;
        if AverMethod,  winX = 3;  else winX = 1; end
    end
    fname = filename(f);
    savedir = sprintf('WSP/Figures/FFT_WinZ_%d/WinX%d_Thr%d_%s/',
                       winZ,winX,MeanThr,fname(10:length(fname)-4));

    % ****************************************
    if LoadWSP == 1;
        load(sprintf('%sWPS_Spectroscopic_analysis.mat',savedir))
    else
```

---

The code snippet above is a MATLAB function that takes input files with spectra and creates a spectroscopic image along with statistics for the size distribution.
fprintf('Processing File: %s\n',fname);
[TD magnTD] = loadTDsignal(f);
[m n] = size(magnTD);
XcorrSegm = zeros(m,n);

bw = im2bw(magnTD,graythresh(magnTD));
bw = bwareaopen(bw, 50);
cc = bwconncomp(bw, 4),
labeled = labelmatrix(cc);
graindata = regionprops(cc,'basic');

% RGB_label = label2rgb(labeled, jet(cc.NumObjects), 'k', 'shuffle');
% figure; imagesc(RGB_label)

imHsv(:,:,1) = 0;
imHsv(:,:,2) = 0;
imMean = zeros(size(magnTD));
MeanSpec = zeros(NP,NQ);
Cp = 0;
Data = [];

% ==============================================================
for p = P;
    Cp = Cp+1
    Cq = 0;

% ==============================================================
for q = Q;
    Cq = Cq+1;
dirname = sprintf(['WSP/MaxPeak/FFT_%dx%d/%s/'...
                    'FFT_%dx%dwin_p%dq%d.mat'],
                  winZ,winX,fname(10:length(fname)-4),winZ,winX,p,q);
if ~exist(dirname,'file'),
    break
else
    load(dirname)
    Data = [Data;Cp Cq Zc Xc 0 0];
spec = (abs(Fxx))./sourceFFT;
    if LPF_ON == 1;
        spec = medfilt1(spec,filterPar1);
        spec = FilterFunction(spec,filterPar2);
    end;
    nKappa = length(spec);
    Sample = spec(remEdgeA+1:nKappa-remEdgeB);
end

nKappa = length(Sample);
SampleCorr_der = autocorr(diff(Sample),nKappa-2);

[y x] = findpeaks(-SampleCorr_der,'MINPEAKDISTANCE',60,'NPEAKS',1);
[y i] = min(abs(CodBw-x));
DiamEst(Cp,Cq) = diamLin(i);
XcorrDer(Cp,Cq) = x;

Data(size(Data,1),5) = DiamEst(Cp,Cq);
if DiamEst(Cp,Cq) > MaxHue;
    disp('DiamEst > MaxHue')
    pause
end
MS = mean(Sample);
Data(size(Data,1),6) = MS;

MeanSpec(Cp,Cq) = MS;
if MS > MeanThr;
    PHue = DiamEst(Cp,Cq)/MaxHue;
    Xa = Xc-DX/2;
    if Xa < 1;  Xa = 1;  end
    Xb = Xc+DX/2;
    if Xb > 500;  Xb = 500;  end
    for z = (Zc-50:min((Zc+50),7000));
        for x = (Xa:Xb);
            if (imMean(z,x) < MS);
                imHsv(z,x,1) = PHue;
                imHsv(z,x,2) = 1;
                XcorrSegm(z,x) = XcorrDer(Cp,Cq);
                imMean(z,x) = MS;
            end
        end
    end
end
%
*---------------------------------------------------------------------*
%
end
%
*---------------------------------------------------------------------*
%
end
%
*---------------------------------------------------------------------*

Magn = 1.1;
imRGB = hsv2rgb(imHsv);
Width = latPixRes(Oversampled+1)*50*Magn;
Height = depPixRes*700*Magn;
figure('units','pixels','position',[900 100 Width Height])
subaxis(1,1,1,'MR',0,'ML',0,'MB',0,'MT',0,...
        'PL',0.02,'PR',0.02,'PB',0.03,'PT',0.02)
imagesc(imRGB); axis off

DX = 500*(0.5/(latPixRes(Oversampled+1)/2));
DZ = 7000*(0.5/7*depPixRes));
x1 = 10*(1+4*Oversampled);
x2 = x1+DX;
z1 = 6900;
z2 = z1-DZ;
line([x1 x2],[z1 z1],'LineStyle','-','color','w','LineWidth',4)
line([x1 x1],[z1 z2],'LineStyle','-','color','w','LineWidth',4)
text(x1+10*(1+4*Oversampled),z1-200,'0.5 mm','color','w','fontsize',12)
caxis([2 MaxHue]);
c = colormap(hsv(60));
c = c(7:60,:);
colormap(c),
colorbar('fontsize',14);
savefig(gcf,'Spectroscopic_image', {'fig'},savedir)

count=0;
clear MovAver Segment A
for i = 1:length(graindata);
    x = round(graindata(i,1).Centroid(1));
    z = round(graindata(i,1).Centroid(2));
    if imHsv(z,x,1) > 0;
        count = count+1;
        Segment(count,1) = i;
        Segment(count,2) = imHsv(z,x,1)*MaxHue;
        Segment(count,3) = graindata(i,1).Area^0.5;
        Segment(count,4) = XcorrSegm(z,x);
        A(count,1) = imHsv(z,x,1)*MaxHue;
        A(count,2) = z;
        % hold on; plot(x,z,'.r','MarkerSize',10); hold off
    % else
    %     hold on; plot(x,z,'.b','MarkerSize',10); hold off
    end
end

PixelEdges = 250:250:7000;
Nd = MaxHue;
DiamCentres = 1:Nd;
B = zeros(length(PixelEdges),Nd);
for i = 1:size(A,1);
    r = ceil(A(i,2)/250);
    c = round(A(i,1)); if c==0; c=1; end
    B(r,c) = B(r,c)+1;
end

figure;
DepthEdges = PixelEdges*depPixRes/1000;
imagesc(DiamCentres,DepthEdges,B); colorbar
set(gca, 'YTick', 1:5, 'YTickLabel', 1:5)
xlabel('Diameter (\mum)');
ylabel('Depth (mm)')
savefig(gcf,'RunningHistogram', {'fig'},savedir)

figure('units','pixels','position',[900 100 350 250])
X = 1:20;
N = hist(Segment(:,2),X);
bar(X,N); % box off
xlabel('Diameter (\mum)');
ylabel('# of spheres')
xlim([0 MaxHue])
set(gca,'XGrid', 'on')
savefig(gcf,'DiameterHistogram',{"fig"},savedir)

DiamEst  = Segment(:,2)';
[y i] = max(N);
Stats(1) = mean(DiamEst);
Stats(2) = std(DiamEst);
Stats(3) = median(DiamEst);
Stats(4) = mad(DiamEst,1);  % Median Absolute Deviation
Stats(5) = X(i);
Stats(6) = fwhm(X', N')/2;

fileID = fopen(sprintf('%sStatistics.txt',savedir),'w.');
fprintf(fileID,'%8s %8s %8s %8s %8s %8s\n',...
'Mean', 'std', 'Median', 'MAD', 'Max', 'FWHM/2');
fprintf(fileID,'%8.1f %8.1f %8.1f %8.1f %8.1f %8.1f\n',Stats);
fclose(fileID);

savefile = sprintf('%sWPS_Spectroscopic_analysis.mat',savedir);
save(savefile);
end
% =============================================================================
break
for i = 1:size(Data,1);
  if Data(i,6) > MeanThr;
    label(i) = text(Data(i,4),Data(i,3),...
       sprintf('(%d,%d)',Data(i,1),Data(i,2)),...
       'color','r','fontsize',12,...
       'HorizontalAlignment','center');
  else
    label(i) = text(Data(i,4),Data(i,3),...
       sprintf('(%d,%d)',Data(i,1),Data(i,2)),...
       'color','y','fontsize',12,...
       'HorizontalAlignment','center');
  end
end
i = 1:size(Data,1);  delete(label(i))
% =========================================================================
% END m-file Create_spectroscopic_image
%============================================================================