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# MINIATURIZED OPTICAL SCATTER IMAGING MICROSCOPE IN REFLECTANCE MODE WITH FOURIER FILTERING FOR MONITORING DEVICES AND CELLS

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# **ABSTRACT OF THE THESIS**

Miniaturized Optical Scatter Imaging Microscope in Reflectance Mode

with Fourier Filtering for Monitoring Devices and Cells

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Optical scatter imaging (OSI), which combines light scattering spectroscopy with microscopic imaging, offers an alternative and efficient way for non-invasive and dynamic study of particle and cell morphology with high signal throughput in real time. A variable diameter iris as a Fourier spatial filter allows the OSI microscope to generate images that encode the intensity ratio of wide-to-narrow angle scatter (OSIR, optical scatter imaging ratio) at each pixel. One part of this study focused on designing and constructing a miniaturized OSI microscope setup adopting reflectance mode with Fourier filtering based on original OSI setup. Recently, the ZnO nanostructure-modified quartz crystal microbalance (ZnOnano-QCM) biosensor has been receiving increasing attention for its dynamic and noninvasive cellular monitoring properties. The goal in this part of the study is to design an optical imaging modality that can be combined with the ZnOnano-QCM

biosensor. The second part of the study is focused on evaluating the performance of this miniaturized OSI microscope setup. Our results demonstrate a compact OSI microscopy system which was build using aspheric lenses, a LED light source, and a low-budget CCD camera. The results show that the microscope aperture of our OSI setup had an NA of 0.55 corresponding to a spatial resolution of 0.6µm, the magnification was 8X, and the field of view was 596 x 446µm. Theoretical simulations of the forward scattered and the backscattered OSIR show that the backscatter OSIR fluctuates more as a function of particle size compared with the forward scatter OSIR which varies monotonically as a function of sphere diameter. When samples were mounted on non-reflective matte black metal slides, the backscattered OSIR of onion cells and microspheres were measured and the experimental data demonstrate that the measured OSIR agrees well with theoretical predictions for microspheres with diameter between 0.7µm and 1.8µm. Onion cell darkfield images on the matte black slides were of good quality and compared well with bright field images collected on a standard phase microscope. However, when samples were mounted on glass or in an aqueous medium with a spatial filter, the acquisition of clear dark-field images of live endothelial cells failed since the spatial filter could not block the specular reflection perfectly. As such, the OSI microscope that was developed provides a proof-of-principle for conducting the OSIR measurement in reflectance mode. However, further improvements are needed to eliminate the background due to specular reflection in samples.

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# Chapter 1

# Introduction

#### 1.1 Motivation

The most fundamental unit of organisms is the cell. With the continuous development of modern molecular biology and the emergence of various new biotechnologies, our understanding of the molecular basis of cell function has progressed considerably. However, much remains to be elucidated about living cells and their internal functioning. Cellular organelles such as mitochondria and lysosomes perform a variety of essential functions in cell physiology but substantial evidence is still needed to reveal their precise roles in many cellular processes, including metabolism, membrane repair, and cell death. One of the main reason given for this in modern biology is the lack of a tool that can monitor cells and subcellular organelles at the submicron level without disrupting their function. The observation of dynamic alteration in the morphology of subcellular organelles is of vital significance in a variety of biomedical applications including clinical diagnosis, drug discovery, and basic cell biology [1]. For example, the most typical feature of precancerous changes is variations in the nuclei of the epithelial cells, such as nuclear enlargement, pleomorphism and hyperchromasia [2]. Therefore, a better technique for observing the morphology of subcellular organelles in real time and in vivo with high resolution can improve the assessment of the normative physiological state and functional

behavior of cells, ultimately leading to more accurate computational models and better diagnostic capability [3].

Conventional optical microscopes have been commonly used for various applications in cellular biology. As compared with histology and other non-optical techniques, optical techniques using visible light are generally noninvasive. However, because of the weak ability of the original cellular structure to absorb visible light, staining (visible or fluorescent) is often employed to enhance optical contrast, which may interfere with normal cell function and confound the results. In order to overcome this limitation, several advanced microscopic techniques have been developed. For example, phase contrast and differential interference contrast (DIC) microscopies take advantage of the principle of phase shifts and interferometry respectively to gain information about the optical phase of the sample. Yet despite the improvement in optical contrast and cells being exempt from staining, the spatial resolution of phase contrast and DIC, just like conventional optical microscopes, is limited by diffraction. In fact, diffraction constrains the spatial resolution of optical microscopy to approximately 1 µm, making it hard to resolve subcellular structures [4]. Since its birth, electron microscopy (EM) has made a significant contribution to modern biology. EM is capable of resolving subcellular structure with very high resolution but impractical for conducting dynamic and real-time study since EM only works with nonfunctional cells [5].

Several super-resolution fluorescence microscopy systems have been developed with

a spatial resolution not limited by the diffraction of light including saturated structuredillumination microscopy (SSIM) [6] and stimulated emission depletion microscopy (STED) [7]. Super-resolution fluorescence microscopy allows dynamic processes to be investigated at the tens-of-nanometer resolution in living cells [8]. While the superresolution fluorescence microscopy has shown the powerful monitoring capacity of subcellular alterations in real time, the optical scatter imaging technique we developed has its own potential advantages. Namely, we can miniaturize the microscope setup to further combine with other monitoring modalities like acoustic monitoring devices and perform the cell analysis with relatively low resolution. The Fourier-filtered images spatially encode the size-sensitive information which directly provides a label-free measurement of local particle size. Furthermore, compared with expensive super-resolution fluorescence microscopes, the cost for our OSI microscope setup is economical.

#### 1.2 Light Scattering

Contrast from elastic scattering provides another option for non-invasive, dynamic study of viable cell function to assess morphology of subcellular particles, whose sizes are close to the optical resolution of microscopes. Compared with the conventional optical microscopes, biosensing and imaging based on light scattering possessing several obvious advantages have recently received significant attention. Light scattering techniques offer much higher signal throughput than modalities based on other contrast because of the large cross sections of the elastic light scattering process. In addition, although image resolution may be still limited by diffraction, signals provided by light scattering exhibit a high degree of sensitivity to subtle alterations in the physical properties of the scattering sources that are below the diffraction limit [1]. For example, when monitoring subcellular organelles by light scattering techniques, changes on nanometer length scales have been reported [4]. More importantly, owing to its unique physical properties, elastic scattering of light at tissue, cellular, and subcellular interfaces is capable of presenting significant contrast for sensing or imaging without the requirement of exogenous markers such as fluorescence. This means that light scattering techniques do not damage the cellular function or structure during imaging and sensing, and then make it possible to study the dynamic changes of cellular structures non-invasively.

Different techniques of utilizing light scattering features were reported. By combined measurements of fluorescence and light scatter, conventional flow cytometry is a widespread and powerful technique for identifying complex cell phenotypes and cellular functions. Moreover, flow cytometry has been combined with fluorescence microscopy to create imaging flow cytometry, where it provides not only quantitative properties but also the bright field and dark-field images of single cells in a population [9]. However, the cell-cell relations are usually lost, since single-cell suspensions are required for all flow cytometry assays. In addition, flow cytometry cannot provide information about the interior subcellular structure of cells, so is not suitable for monitoring subcellular morphological alterations in cells. Another important technique is light scattering spectroscopy (LSS)

which is capable of quantitative characterization of cellular and subcellular morphology without destroying viability. After rapid development in recently years, polarized [10], angular-dependent [11-13], and wavelength-dependent [14, 15] LSS has been constantly improving and applied in many fields. Although LSS has a great advantage in noninvasive monitoring of subcellular morphology, to validate the scattering sources contributing to the signal, one must compare the measured data with the sample image captured separately to infer the location of the incident beam or to restrict this beam to a specific microscopic region of the sample.

Furthermore, a few advanced microscopic visualization techniques, including optical coherence tomography [16] and confocal light absorption and scattering spectroscopy (CLASS) [17], derive contrast from optical scattering. In addition, Dzakpasu *et al.* verified the applicability of dynamic light scattering microscopy to image submicroscopic motions of polystyrene beads and living macrophage cells [18]. And Ho *et al.* used angle-resolved low coherence interferometry measurements of nuclear morphology for detecting cervical dysplasia [19].

#### **1.3 Initial Optical Scatter Imaging Developed in Our Laboratory**

In this study, we also use a light scattering technique that relies on angular dependent light scattering called optical scatter imaging (OSI) to study particle and cell morphology. OSI is a technique which combines light scattering spectroscopy with imaging microscopy that can non-invasively monitor subtle morphological alterations in particle with wavelength-

scale dimensions without any fluorescent dyes or organelle isolation. OSI has been employed to detect and track mitochondrial morphology changes [20, 21]. Furthermore, an improved OSI system with a digital micromirror device (DMD) capable of characterization of particle shape and orientation was developed [22].

The current OSI microscope setup is designed based on the initial OSI system developed in our laboratory. The working principle and initial system design of OSI were described in detail previously [23]. Briefly, the primary task of the initial OSI system is to monitor the intensity ratio of wide-to-narrow angle scatter (OSIR, Optical Scatter Image Ratio). To achieve this, a variable iris with a center stop placed in a plane conjugate to the objective's back focal plane collected light scattered within a solid angle, and the iris diameter was manually adjusted between either a low NA ( $3^{\circ} < \theta < 10^{\circ}$ ), or a high NA  $(3^{\circ} < \theta < 67^{\circ})$  position, controlling the angular spread of the field detected in the imaging plane. For each sample, two sequential dark-field images were acquired at high and low NA respectively and then the scatter ratio image was generated by dividing the background subtracted high NA image by its corresponding background subtracted low NA image. Such an image spatially encoded the high-to-low NA optical scatter image ratio (OSIR) at each pixel in the field of view. In addition, previous study showed that measured optical scatter image ratio (OSIR) declines monotonically as the diameter of the scattering particles increases, making this ratio an indicator of particle size [23]. Therefore, the scatter ratio image can be used to detect the alterations in the size of particles.

However, in comparison to fluorescence, OSI generates data characterizing the structure of sample which is not molecularly specific. In addition, the structural data does not strictly correspond to a specific source in the process of imaging. Consequently, it is necessary to employ other techniques, such as fluorescence or DIC, to validate initially the specification of the results. Once this validation is completed, then the label-free OSI method can be used to monitor a given process.

#### 1.4 Fourier Filtering

OSI can be considered effectively an analog Fourier filtering technique [23]. During the process of OSI, before the ratio of high and low NA images is calculated, signals are spatially filtered by the variable iris. Such an iris is used as a circular Fourier filter designed to allow only specific angles of scatter to pass. Several Fourier-filtered images generated by measuring the OSIR enable researchers to extract the sample's angular scattering properties at each pixel within the full field of view [1]. In contrast to conventional optical microscopes, the scatter ratio image encoded the OSIR maps the sample by directly providing quantitative information pixel by pixel which characterizes particle size at every point within the field of view. Sensing based on these quantitative maps not only does not require the user to distinguish particles under the microscope but also provides superior signal throughput, providing a more detailed and straightforward way to observe particle morphology. Furthermore, due to Fourier filtering of scattered light, the OSI technique remains highly sensitive to subtle alterations in object size below the limitation of pixel size.

The previous results showed that Fourier filter can in effect extend the assessment of sample morphology, and have more application potential. Moreover, in order to select different scattering parameters, different Fourier filters could be designed. For example, Gabor-like filters which excel at characterizing particle orientation and roundness have been implemented in microscopy for measuring subcellular texture [24] and sorting particles [25]. A technique based on Gabor-like filters has the advantage that it does not rely on Mie theory, so it is applicable to characterize morphology of non-spherical organelles or particles.

#### 1.5 ZnO Nanostructure-modified QCM Biosensor

The ZnO nanostructure arose as a new material receiving great attention within biosensor technology because of its proven biocompatibility and distinctive sensing ability [26, 27]. Meanwhile, the quartz crystal microbalance (QCM) which is an acoustic wave device has been widely applied as a biosensor for cell monitoring based on impedance spectrum analysis [28, 29]. Recently, combining ZnO nanostructures and QCM, Reyes *et al.* developed a ZnO nanostructure-modified quartz crystal microbalance (ZnOnano-QCM) biosensor in which dynamic and noninvasive monitoring of the adhesion and proliferation of bovine aortic endothelial cells (BAEC) was successfully achieved inside a standard cell culture environment [30]. Another important property of such novel biosensor is its micro volume, so it can be readily deployed inside a cell-culture well monitoring cell activities in real time. However, it suffers from producing signals derived from the total changes of the

measured specimen load on the sensor, unable to direct at a single cell. The use of an optical imaging modality as a complementary method might be a good option for filling this gap.

Based on the initial OSI system, the current OSI microscope setup is designed and constructed so as to be miniaturized and combined with the ZnOnano-QCM biosensor for enhancing its performance in the future. The optical mode enables correlation of the macroscopic viscoelastic mechanical changes measured by acoustic mode with morphological dynamics occurring at the single cell and subcellular scale. Eventually, this OSI microscope setup can be ultimately deployed with the ZnOnano-QCM biosensor to create a compact and integrated system performing both acoustic and optical monitoring inside a standard incubator where the cell will be cultured and monitored simultaneously. With the miniaturized OSI microscope setup in the incubator, one can continuously monitor cellular processes in real time. This miniaturized OSI microscope setup can also be used independently in other biological applications. It has great potential in continuously and noninvasively monitoring subcellular morphology or observing the effects of various drugs on cell and organelle function.

#### **1.6 General Outline of This Thesis**

In what follows, the thesis is organized into three chapters. In Chapter 2, we will introduce the design, material selection, and construction features of our miniaturized OSI microscope setup. In Chapter 3, we will show how the performance of this OSI microscope setup is evaluated. With the new OSI setup, we will demonstrate the feasibility of two different methods on obtaining dark-field images and measuring the backscattered OSIR of microspheres with different diameters. The microscope setup will be also used to image the BAEC maintained in an aqueous medium. In Chapter 4, we will present our conclusions and give our plans for future improvements.

# **Chapter 2**

# Microscope Setup Design and Construction

#### 2.1 Introduction

The initial OSI setup is a transmission microscope employing the central dark-ground method. Through a center stop optically blocking transmitted light, each pixel value of the images is in direct proportion to the amount of scattered light from the particles within a target object. In this study, the current setup we designed and constructed based on the original OSI setup was a miniaturized microscope adopting reflectance mode with Fourier filtering. The microscope setup should be made sufficiently small and lightweight to be ultimately placed in the standard humidified incubator. Therefore, besides generating highresolution images, miniaturization is an important consideration. In order to facilitate miniaturization, we gave up using conventional microscope objectives because their operation needed the use of a coordinated tube lens with a focal length of 200 mm which would make the device oversized. Instead, 8x magnification was achieved by a designed lens system consisting of four aspheric lenses with each focal length less than 20mm. In this chapter, we will demonstrate a compact OSI microscopy system which was build using aspheric lenses, LED light source, and a low-budget CCD camera.

#### 2.2 Equipment

To build a compact and portable prototyping optical setup, 16mm and 30 mm cage

systems including rods and mount plates provided by Thor labs, NJ, were used. One of the assembly cage rods (ER4E, Thor Labs, NJ) featured an engraved scale with increment of 1 mm along the side, allowing accurate adjustment of distances between aspheric lenses according to their focal lengths. In order to get better image resolution, aspheric lenses, instead of conventional spherical lenses, were used because of their ability to correct spherical aberration. Specific to the reflectance mode is a non-polarizing beamsplitter cube (BS010, Thor Labs, NJ) to reflect light downward to the specimen and transmit the reflected (backscattered) light upward. Other standard components included two cage system iris diaphragms (CP20S, Thor Labs, NJ), a LED light source (Cree XP-E2, LEDSupply, VT) with 535nm wavelength, and a CCD camera (BFLY-U3-13S2C-CS, Point Grey Research). The two cage system iris diaphragms possess continuously variable apertures, Ø0.8 mm to Ø20.0 mm, adjusted by a button head lever. The CCD camera contains a rectangular 1288 x 964 pixel CCD element, with each pixel size 3.75 x 3.75µm. The corresponding output images have the same pixel resolution. With an object of known size, the magnification of the setup can be easily calculated by comparing the image size on the CCD element and the actual object size. Key components of the optical setup, such as aspheric lenses and CCD camera, were pre-mounted into their corresponding cage plates and then integrated into the cage system aligned along a common optical axis.

For sample mounting, a special sample stage was made by 3D-printing using polylactic acid (PLA) plastic as shown in Figure 1. The CAD model was designed in the browser based program Tinkercad (tinkercad.com) and the fabrication was conducted at the Rutgers Makerspace printing lab by Adeeb Jacob Hanna who was an undergraduate student and assisting me. At the top, the rectangular holder with C clamp shape can perfectly mount a standard microscope slide with size 25mm × 75mm. In the bottom, the hollow cylinder with a diameter Ø23.50 mm was embedded in a Z-Axis translation mount (SM1Z, Thor Labs, NJ). The Thor Labs Z-Axis translation stage held the 3D-printed sample mount using its retaining rings and provided precise and fast movement in Z direction making it convenient for bringing the image of the object into focus on the CCD camera. A cage alignment plate (CPA1, Thor Labs, NJ) was used to help simplify the alignment process during optical setup construction.



**Figure 1.** The CAD model of the 3D-printed sample stage. The rectangular holder has dimensions 3mm x 27.5mm x 25.5mm and the hollow cylinder has a diameter of 23.50mm and a height of 7mm.

#### 2.3 Considerations for the Microscope Setup Design

First, according to the model specifications the CCD camera (BFLY-U3-13S2C-CS) has the highest quantum efficiency of 46% for green light (at 525nm) whereas efficiency for blue (43%, at 470nm) and red (40%, at 640nm) light are relatively less. Hence, a light source employing green light is preferable for efficient excitation. Second, it is more beneficial to use a high numerical aperture (NA) aspheric lens for efficiently collecting and detecting the scattered light emitted by specimen. Collimated light is incident on the specimen and the light is then reflected and backscattered from the surface with the scattering angles spreading out. The wider the range of angles accepted by the lens, the more these scattered angles are able to be collected by the lens, and the more information it contains to resolve finer details. And with the capture of more orders of diffraction emitted from the specimen, it is also expected that high NA lens will collect more light generating brighter images, which is particularly valuable in reflectance mode where the classic image is a bright object against a dark background. Third, the capability of conducting spatial frequency filtering in the Fourier transform plane is a critical design and application requirement for this OSI setup. There needs to be enough space between two aspheric lenses for positioning a Fourier filter, like a dark-field filter, and its corresponding mounts.

#### 2.4 Microscope Setup

The current optical scatter imaging setup in reflectance mode is shown in Figure 2. The specimens were mounted on the 3D-printed sample stage. A CCD camera (BFLY-U3-13S2C-CS) was positioned at the top of the setup and connected to a PC. Sample illumination was achieved with a green LED light source (Cree XP-E2, 535nm, LEDSupply, VT), which was limited by a closed iris diaphragm (Ø0.8mm) to simulate a point light source. The divergent light was collimated by an aspheric lens, L0, with focal length 11mm and then passed through a non-polarizing 50:50 beamsplitter cube (BS010, Thor Labs, NJ) which was mounted in a 16mm cage cube (SC6W, Thor Labs, NJ). With the help of the beamsplitter, the collimated incident light was reflected downward and directed onto the specimen by passing through a pair of aspheric lenses, L2 (C280TMD-A, f = 18.4mm, NA = 0.15, Thor Labs, NJ) and L1 (C230TMD-A, f = 4.51mm, NA = 0.55, Thor Labs, NJ),

which was aligned in Köhler illumination to allow even illumination of the sample. In addition, L1 was also used to capture and collimate the light backscattered by the specimen. After re-entering L1 and L2 and passing through the beamsplitter in an upward direction, the scattered light passed into another pair of aspheric lenses, L3 (C240TME-A, f = 8mm, NA = 0.5, Thor Labs, NJ) and L4 (C260TMD-A, f = 15.3mm, NA = 0.16, Thor Labs, NJ), where it was finally directed to the CCD camera. If the sample is transparent, most transmitted light will pass through the sample. However, there is still a small amount of light reflected at the glass surface of the sample chamber. After returning to the lenses and passing through the beamsplitter, the back reflection of the incident beam will be blocked by a spatial filter consisting of a central plastic "dot" affixed to a coverslip at the back focal plane of the L3. However, the higher angles of backscattered light will not be blocked by this center stop.

Four aspheric lenses, L1-L4, were used to control the amount of magnification and to focus the specimen's image on the CCD camera in this design, while L1 and L2 also served as delivering deflected incident light to the specimen. L1 and L2 were the first part of this lens system, forming a primary, enlarged image of the specimen. L3 and L4 were the second part which formed a secondary, further enlarged image. The final magnification is given by the following equation

$$M = \frac{f_2}{f_1} \times \frac{f_4}{f_3}$$

where  $f_1-f_4$  are the focal lengths of L1-L4 respectively. Thus the final magnification of the OSI setup should be X7.8 in theory. The microscope aperture of this OSI setup is determined by the NA of L1 and expected to equal 0.55 since L1 is the first lens within the microscope system to capture and collimate the backscattered light from the specimen.



Figure 2. (A) Schematic diagram of the current miniaturized OSI microscope setup in reflectance mode. The transmitted light (blue beam) is used to illuminate the specimen. A closed iris diaphragm is positioned in front of the LED to simulate a point source and L0 collimates the light. BS, 50:50 beam splitter, is mounted in the center of the cage cube. Four aspheric lenses (L1-L4) capture the scattered light (red beam) from the specimen and bring the image into focus on the CCD camera. A center stop may be placed at the Fourier plane to block the specular reflection off the sample surface. Fourier filtering may also be done in the back focal plane of L3. The sample slide is mounted on the sample stage. (B) Photograph of the current miniaturized OSI microscope setup in real life.

#### 2.5 Conclusion

A reflectance-mode OSI microscope setup was constructed with a lens system consisting of four aspheric lenses (L1-L4) as its only magnifier. The position of each aspheric lens was adjusted based on each focal length to bring the image of the object into focus on the CCD camera. The magnification of the real and enlarged images is expected to be 7.8X, which needs to be experimentally determined. The microscope aperture of the OSI setup is expected to have an NA of 0.55. Further validation also needs to verify whether the microscope aperture of the OSI setup is not cut off. The performance of this OSI microscope setup will be evaluated and described in the next chapter.

# Chapter 3

# Results to-Date

#### 3.1 Measurement of the Magnification, Field of View and Resolution

To investigate the setup performance, we measured the magnification, field of view and spatial resolution of the new OSI microscope setup.

#### 3.1.1 Materials and Methods

To measure the specifications of the microscope setup, a standard stage micrometer specially designed for reflective light (2269-10, Ted Pella, Redding, CA) was used as a reference object. The stage micrometer is 1mm total length and is divided in 100 parts, which means each division is 10µm across. Instead of a glass substrate, its scales were etched on a highly reflective vacuum coated metal, appearing black against a bright background on the image under reflectance mode. The illumination employed the greenlight LED (535nm). Images were captured based on the arrangement of the microscope setup shown in Figure 2 without any filtering, and the CCD camera was positioned at the back focal plane of L4 to collect the real image of the object. The output image size can be measured by counting pixels. The true value of the magnification of the microscope setup can be calculated by comparing the image size on the CCD camera and the known size of the object. According to the real distance corresponding to every pixel, the field of view can also be easily obtained from the known resolution of the image. The resolution of the setup was measured in the Fourier domain by measuring the number of diffraction orders passed by the stage micrometer with known line spacing of 10µm.

#### 3.1.2 Image Capture and Analysis

The software for image acquisition was FlyCap2 viewer 2.7 (Point Grey Research). A bright field image of the Ted Pella stage micrometer captured by the CCD camera is shown in Figure 3. Its pixel count was implemented in the open source image processing software Fiji 1.0 (http://fiji.sc/). As shown in Figure 3, we measured the distance between scale lines of 50 and 80 in pixels with Fiji software and the result was 642.234 pixels across. Since each pixel size is 3.75µm x 3.75µm, the distance was 2408.38µm on the CCD. The true magnification is obtained by dividing the image size by the object size and the value is 2408.38/300, or 8X. Furthermore, knowing the extent of the camera in pixels, the field of view of the microscope setup can be obtained as approximately 596 x 446µm.

The image resolution of the microscope setup can be theoretically predicted based on the radius of the Airy diffraction pattern the microscope setup creates. The Airy pattern takes place when light passes through a circular aperture. The Airy pattern radius,  $r_{airy}$ , is given by the following formula

$$r_{airy} = \frac{1.22\lambda}{2NA},$$

where  $\lambda$  is the wavelength of the incident light and *NA* is the objective numerical aperture. In the following Section 3.2, we experimentally validated that the microscope aperture of our OSI setup was not cut off. Therefore, the objective numerical aperture is determined by the numerical aperture of L1, 0.55. The wavelength of the incident light,  $\lambda$ , is 535nm. The theoretical spatial resolution of the microscope setup, *D*, can be derived as

$$D = \frac{0.61\lambda}{NA} = \frac{0.61 \times 535nm}{0.55} = 0.593 \ \mu m \approx 0.6 \ \mu m$$

With the microscope setup magnification 8X, the camera's pixel resolution is magnified as roughly 4.7µm in the output image which is larger than the pixel size of the CCD camera 3.75 x 3.75µm. The pixel size is not a limiting factor in the measurement of the OSIR which is not limited by pixel resolution. However, for acceptable sampling, the smallest resolved element, 0.6µm, must be sampled by at least two pixels. Therefore, the magnification should be about 12.5X to get images at the resolution capability of the microscope, which can be improved in the future. Furthermore, the spatial resolution can be precisely measured using a standard resolution target or by obtaining the point-spread function with a 0.3µm microsphere image.

In Section 3.5, we measured the ratio of wide-to-narrow angle scatter, the OSIR, of microspheres with diameter 0.356, 0.771, 0.989, 1.826µm, in which only the measurement of microspheres with diameter 0.356µm showed a great deviation from the theoretical prediction. It illustrates that particles with diameter 0.771, 0.989, 1.826µm can be resolved by such microscope setup.



**Figure 3**. Image of the standard Ted Pella stage micrometer captured in bright field reflectance. The yellow line highlights the distance with a known length of 300µm which is used to calculate the true value of the microscope setup's magnification.

### 3.1.3 Conclusion

The true value of the magnification of the microscope setup is 8X, which agrees well with the theoretical value, 7.8X. The field of view of the setup is 596 x 446µm. The theoretical spatial resolution is calculated as approximately 0.6µm. Particles with diameter 0.771, 0.989, 1.826µm can be resolved by measuring the OSIR, which is detailed in Section 3.5.

#### 3.2 Investigation of Diffraction Pattern

When illuminated, the sample emits a diffraction pattern with orders of diffraction spreading. For example, one of the classic diffraction patterns is the Airy disk resulting from uniformly illuminating a circular aperture. The second experiment we designed and conducted to test our OSI setup was the investigation of the diffraction pattern of a standard stage micrometer, which acts like multiple slits with 10µm spacing. If the optical system cannot collect all the orders of diffraction as expected, this microscope setup will not collect all the scatter angles that we can and the corresponding resolution of the setup will suffer. With the diffraction pattern investigation, we validated that the microscope aperture of this OSI microscope setup was not cut off.

#### 3.2.1 Material and Methods

To evaluate the performance of the microscope aperture, the same Ted Pella standard stage micrometer for reflective light was used. Since the scales are etched precisely at an equal distance along the line, the stage micrometer can be seen as a diffraction grating with 100 lines/mm. Based on the comparison of the theoretical calculation to the maximal order of diffraction and the experimental results of the total number of spots which can be obtained in the Fourier plane in practice, we can verify whether the microscope aperture is not cut off. Furthermore, the measurement of the distance between the zeroth order spot and the first order spot provided the practical data for the fabrication of high and low

frequency filters in the next part.

In order to reveal the diffraction pattern of the stage micrometer and count the diffraction order, it was necessary to obtain images of the Fourier plane. For illumination, the same green-light LED (535nm) was used. Since the size of spots in diffraction pattern was governed by the size of the point source, the iris diaphragm in front of the LED was closed to the minimum (Ø0.8mm). The stage micrometer was first fixed onto the sample stage and the distance was adjusted between the stage micrometer and the collection lens, L1, by the Z translation stage until the image was in focus. To capture the Fourier plane image, the same CCD camera was employed with the help of one aspheric lens, L4 (f=15.3mm). However, instead of positioning the CCD camera at the real plane collecting the image of the object like our original setup, we repositioned the location of both the CCD camera and L4 until the image formed at the rear focal plane of the L3 (f=8mm), the Fourier plane, was brought into focus on the CCD camera. In addition, a Thor Labs reflective neutral density (ND) filter with 1% transmission was placed in front of the LED light source adjusting the intensity of light to avoid the over-saturation of the detected signal on the CCD.

#### 3.2.2 Theoretical Calculation

According to the grating equation for normal incidence, we can calculate the order of diffraction by use of the following formula:

$$m=\frac{d\sin\theta_m}{\lambda},$$
where *d* represents the slit spacing,  $\lambda$  represents the wavelength of the incident light and  $\theta_m$  represents the angle between diffracted ray to the grating's normal vector. Since the maximal half-angle of the cone of light,  $\theta$ , that can be collected by the lens is determined by the lens's numerical aperture (NA), the equation for the maximal  $\theta_m$  is derived as

$$NA = n \sin \theta_m$$
,

where *n*, index of refraction of the air, equals 1.00. The wavelength of the light source,  $\lambda$ , is 535nm. The numerical aperture of L1 is 0.55. The distance from one slit to its adjacent slit on the micrometer scale is fixed as 10µm. By combing the two equations, the maximal order of diffraction can be derived as

$$m_{max} = \frac{10\,\mu\text{m} \times 0.55}{535\,nm} = 10.03 \approx 10.$$

Therefore, theoretically the maximal order of diffraction of specimen generated by our OSI setup is 10th. The theoretical results predict that we should obtain 10 spots including both light and dark spots on each side of the center bright spot (the zeroth order) in the Fourier plane image.

#### 3.2.3 Image Capture and Analysis

The image of the diffraction pattern of the stage micrometer is shown in Figure 4. An array of spots including a central bright spot was recorded under the presence of a ND filter with 1% transmission. With the help of the ND filter, the spots from the high orders of diffraction can be clearly observed, whereas the spots from low orders are somewhat hazy and dim. We added some additional contrast to the image using the software Fiji to help clearly

display all spots from both low orders and high orders of diffraction, causing the background in the image to also became a little brighter. By analyzing the Fourier plane image shown in Figure 4, we can accurately count that there are 10 spots in total in each side of the center bright spot including 8 light spots and 2 dark spots located at 4<sup>th</sup> and 8<sup>th</sup> order, which meets the theoretical calculation.



**Figure 4**. Image of the diffraction pattern of the Ted Pella stage micrometer captured using greenlight illumination (535nm) with the help of ND filter with 1% transmission. The brightness of the image was adjusted by the software Fiji. The yellow numbers highlight a total number of 10 light and dark spots on each side of the center bright spot.

## 3.2.4 Conclusion

As shown in Figure 4, 10 spots corresponding to 10 orders of diffraction on each side of the center bright spot in total are observed as expected. Comparing the theoretical and experimental results, it is concluded that the microscope aperture of our OSI setup is not cut off. The arrangement of the setup itself will not decrease resolution and allow us to collect all the scatter angle that we can.

#### 3.3 Acquisition of Dark-field Images Using A Dark-Field Filter

To verify the feasibility of collecting dark-field images and blocking specular reflection in the microscope setup, a dark-field filter consisting of a central plastic "dot" affixed to a coverslip was placed at the rear focal plane of L3 to block the specularly reflected light and perform Fourier filtering. Several dark-field filters with different sizes were manually fabricated. The same Ted Pella stage micrometer for reflective light was imaged as a test sample. Dark-field images were acquired, analyzed, and compared with bright-field images to evaluate the performance of the microscope setup.

## 3.3.1 Material and Methods

As shown in Figure 5, a center stop serving as dark-field filter was positioned at the rear focal plane of the L3, so that the incident light which underwent specular reflection at the sample surface can be blocked. More importantly, since the rear focal plane of the L3 was the Fourier plane, the center stop also performed Fourier filtering to the scattered light

from the sample by removing zeroth order and first order of diffraction.

The dark-field filter was created by adding blackout dots onto a coverslip using NinjaFlex TPU flexible filament with black color (MPN: 85461100600, Fenner Drives). The fabrication was conducted by Adeeb Jacob Hanna who assisted me and the fabrication procedure was as follows. A laboratory hotplate was turned on and the coverslip was thoroughly cleaned. A length of 1.75mm TPU filament was cut and a razor blade was used to cut a thin slice from one end (<0.5mm) resulting in a coin shaped piece of TPU. The blade was used to also cut down the edges of the coin shaped TPU until a smaller circle of desired size was obtained. The desired filament piece was placed onto the clean coverslip and the coverslip was heated on the hotplate until the filament piece melted into a dot. The coverslip with the melted dot was removed and left to cool. Finally, several darkfield filters with different sizes were fabricated including Ø0.53mm, Ø0.60mm, Ø0.69mm, Ø0.72mm, and Ø1.15mm. The distance between two first order spot on each side was measured to be roughly 0.3mm based on the image of the diffraction pattern of the stage micrometer. To avoid blocking higher order spots, the dark-field filter with the smallest diameter, Ø0.53mm, was selected to use as shown in Figure 6.



Figure 5. Image of placing the dark-field filter ( $\emptyset$ 0.53) in the rear focal plane of L3 to block the

specular reflection and perform Fourier filtering.



Figure 6. Image of the blackout dot of the dark-field filter with a diameter of 0.53mm photographed

in the Zeiss Axiovert 40 CFL (X10 magnification).

#### 3.3.2 Image Capture and Analysis

Images A and B were taken by the microscope setup under bright-field and dark-field conditions respectively as shown in Figure 7. In (B) the dark-field filter blocked out the zeroth order spot and first order spots, stopping their corresponding spatial frequencies from reaching the image plane. Presumably, since the sharp edge detail between bright and dark regions of the image is governed by the high spatial frequencies, the removal of the first order spot will lead to minimal loss in image resolution. Additionally, following by the removal of the zeroth order spot centered in the transform plane, regions that were originally black in the image are now expected to become bright, while regions that were white are expected to appear dim. Moreover, the directly transmitted light from the specular reflection off the sample surface does not reach the image plane due to the dark-field filter block, turning the bright background of the image into a dark background.

In (B), the scales of the stage micrometer became bright against a dark background after Fourier filtering as expected. However, unlike scales in (A) with sharp edges, the edges of the scales in (B) seem to be less sharp and the white mark lines contain some black artifacts. Furthermore, in (B) there is apparent light leakage in the top left corner, showing that the specular reflection was not perfectly blocked. These drawbacks may result from the irregular circular shape of the dark-field filter as shown in Figure 5 that cannot block the specularly reflected light perfectly. The drawbacks may also derive from the LED light source which is difficult to collimate and will result in a poor focus at the center of the Fourier plane.



**Figure 7.** Images of the Ted Pella stage micrometer taken by the microscope setup using greenlight illumination (535nm). (A) Imaging in bright field without any filtering. (B) Imaging in dark field with a center stop at the rear focal plane of L3.

With the dark-field filter we fabricated, we successfully filtered the zeroth order of diffraction turning the original black regions of the image into bright regions over a dark background. However, the shape of the center blackout dot of the dark-field filter is not perfectly rounded. The sharpness and resolution of the image are less than expected and there is still some light leakage from transmitted light.

#### 3.4 Acquisition of Dark-field Images Using Matte Black Metal Slides

In this section, we employed another method to acquire dark-field images without placing a dark-field filter. The method is to use matte black metal slides (813-535, Ted Pella, Redding, CA) for sample mount. Just like metallographic microscopy, the light will not reflect from the metal slide and with the matte black surface, the target sample will appear bright against a black background on the CCD camera, allowing dark-field imaging. Onion skin epidermal cells were imaged as a test sample.

## 3.4.1 Materials and Methods

The Ted Pella matte black metal slides are made from matte black anodized aluminum providing reduction in surface light reflections. We obtained the onion epidermal cells from one layer of onion bulb as follows. A medium size onion was cut laterally into several pieces. A scale leaf was removed from one of these pieces. The scale leaf was broken in the middle towards the shiny side and the two pieces were very gently peeled apart. There was a thin transparent layer of cells holding these two pieces of the onion layer together which was the layer of onion epidermal cells we needed for the specimen. A small portion of such thin and delicate epidermis was peeled and placed on the matte black metal slide. Finally, the metal slide was mounted with the specimen onto the microscope setup for imaging without any additional filtering. Green-light illumination (535nm) was used and settings of image capture software remained the same as in the previous section. Moreover, to evaluate the dark-field images of the onion skin epidermal cells, a glass slide with mounted onion epidermal cells was also prepared and cells were observed and photographed under an inverted microscope with magnification 10X (Axiovert 40 CFL, Carl Zeiss, German) for comparison.

#### 3.4.2 Image Capture and Analysis

Images of onion skin epidermal cells captured by the OSI microscope setup and the Zeiss Axiovert inverted microscope are shown in Figure 8. A transparent thin layer of onion epidermal cells attached to the slide surface and its microscopic structure gave rise to light scattering. The matte black metal surface helps stop most of the specular reflection. Therefore, in (A) the edges of the cells can be clearly observed and appears bright against a dark background. Compared with cell edges in (B), in (A) cell edges seem to be sharper and more distinct, providing a higher contrast. However, there are some bright flares in (A) that may result from the uneven surface of the onion cell specimen.



**Figure 8.** Images of the onion skin epidermal cells. (A) Imaging in dark field without any filtering using matte black metal slides. (B) Imaging by Zeiss Axiovert inverted microscope with magnification 10X.

## 3.4.3 Conclusion

We successfully acquired dark-field images of onion epidermal cells using matte black metal slides. Distinct edges of onion cells can be observed in the dark-field images.

#### 3.5 Validation of OSIR Measurements Under Dark-field Condition

The initial OSI setup was used to monitor the optical scatter image ratio (OSIR) using transmission mode in central dark-field microscopy. [23]. Here we validated the feasibility of measuring the backscattered OSIR of microspheres with different diameters and onion skin epidermal cells based on the method in the previous section where dark-field images were generated using non-reflective matte black metal slides. According to Mie theory, theoretical calculation of the backscattered OSIR of microspheres was conducted for comparing with practical values from experimental measurement. Since our setup is in reflectance mode, the OSIR is derived from the backscattered light. To compare with forward scatter in transmission mode, the forward scattered OSIR of spheres with same diameter was also theoretically calculated. To investigate how a lower index ratio m affects the backscattered OSIR measurement, the simulations of both the forward and the backscattered OSIR as function of particle diameter for index ration m=1.04 were performed.

#### 3.5.1 Materials and Methods

The same green LED light source (535nm) was used for illumination. To generate scatter

images under high-NA and low-NA conditions, a variable circular iris (CP20S, Thor Labs, NJ) was inserted as Fourier filter into the back focal plane of L3 performing Fourier filtering. This circular iris was manually adjusted to be closed ( $0^{\circ}<\theta<10^{\circ}$ ) for low NA filtering or open ( $0^{\circ}<\theta<34^{\circ}$ ) for high NA filtering. Based on the grating equation, a solid angle,  $\theta=10^{\circ}$ , for collecting scattered light can permit 3 orders of diffraction to pass through. The diffraction pattern of the Ted Pella stage micrometer was imaged with iris filter closed using the method detailed in Section 3.2. As shown in Figure 9, the edge of the iris aperture was roughly located in the middle of spots of 3<sup>rd</sup> and 4<sup>th</sup> diffracted orders. It is validated that scattered light can be collected within 0° -10° using the smallest diameter of the iris aperture, Ø0.8mm. When the iris is open for the high NA filtering, the biggest angle of collection of scattered light,  $\theta=34^{\circ}$ , is determined by the NA of L1, NA=0.55.

Monodisperse polystyrene microspheres (Polysciences, Warrington, PA) with refractive index = 1.6 were mounted on the Ted Pella matte black metal slides for measuring OSIR. The coefficient of variance (CV) of 0.771µm and 0.989µm microspheres is 3% and the CV of 0.356µm and 1.826µm microspheres is 5%. To avoid stacks and allow individual microsphere to be observed, the original Polysciences aqueous suspensions of polystyrene microspheres were diluted to lower concentrations by deionized water, 1:60. After homogenizing the solutions using a vortex mixer, a 10µL diluted aqueous suspensions of polystyrene microspheres was added onto the surface of a matte black metal slide and left to dry at room air. Without water, the movement of the

microspheres stopped after drying, keeping the microspheres stationary during imaging. Coverslips were omitted because their glass surface will increase specular reflection. Microspheres with diameter 0.356, 0.771, 0.989, 1.826µm were used to perform OSIR measurement.

For each specimen studied, two sequential images of the surface of the matte black metal slide without microspheres were taken at high and low NA as high and low NA background signal respectively by manually adjusting the circular iris. Then two sequential dark-field images of the specimen were also captured at high and low NA. When imaging, the automatic brightness adjustment in the image acquisition software FlyCap2 was disabled. The background, high NA, and low NA images of microspheres with diameter 0.356, 0.771, 0.989, and 1.826µm were stacked respectively in the software Fiji. A custom written Fuji plugin was used to automatically align the images accurately (https://sites.google.com/site/gingzongtseng/template-matching-ij-plugin), correcting the misalignment among images due to the manual adjustment of the iris diaphragm. These aligned images were imported into Matlab, where the acquired raw data was converted to double precision. Images encoding OSIR at each pixel were generated by dividing the background subtracted high NA image by the background subtracted low NA image. Note that the negative values caused by the background subtraction were set to zero. In addition, the infinite value, inf, and the complex value, NaN, caused by high and low division were set to zero as well. To compare with theoretical prediction, pixel histograms

showing the distribution of OSIR value were obtained from the OSIR images. The background of the OSIR images contains a very large number of pixels with OSIR value 0. Therefore, the data with OSIR value ranging from 0 to 1 are omitted when generating histogram images. Also note that theoretically the OSIR cannot have a value less than 1. As such, OSIR values under 1 likely represent noise values.

For the OSIR measurement of onion cells, two sequential images of onion epidermal cells mounted on the matte black metal slide were taken at high and low NA by manually adjusting the circular iris. By dividing the onion cell high NA image by its corresponding low NA image, the onion cell OSIR image was generated.



**Figure 9.** Image of the diffraction pattern of the Ted Pella stage micrometer using the smallest iris aperture, Ø0.8mm. Three spots of high orders of diffraction in each side of central bright spot can be observed.

#### 3.5.2 Theoretical Calculation and Analysis

For spheres, the light scattering intensity is governed by three parameters: the wavelength  $\lambda$  of incident light, the size parameter *x*, and the refractive index ratio *m*. The size parameter *x* and the refractive index ratio *m* are defined as

$$x = \frac{\pi n a}{\lambda}, m = \frac{n_1}{n},$$

where *a* represents the diameter of the spheres, and  $n_1$  and *n* are the refractive indices of particle and surrounding medium respectively. To calculate the scattered light intensity in

all scattering directions, it is critical to solve for the scatter intensity phase function  $F(\theta, \phi)$ . For spherical particles with arbitrary refractive index, Mie theory can be used to derive the phase function  $F(\theta, \phi)$  [31]. Solving for  $F(\theta, \phi)$  involves utilizing Maxwell's equations to calculate the electromagnetic fields propagating inside and outside the particle. One opensource program, MieTab, has been created by August Miller of New Mexico State University to solve for Mie theory and perform simulations of light scattering or light absorption by homogenous spheres (http://amiller.nmsu.edu/mietab.html). However, in the general case of a scatterer with arbitrary shape and refractive index, elements in Solving for  $F(\theta, \phi)$  are rarely solved analytically. Instead, an approximation is usually made to simplify the problem depending on the biological system at hand. For example, Graaff et al. presented a simple numerical approximation of Mie scattering for 1<m<1.1 [32] and Ovryn et al. demonstrated a model for predicting the scattering of a plane polarized wave by a spherical particle with a high numerical aperture (NA) microscope [33].

Here, the wavelength  $\lambda$  of incident light is 535nm. The size parameters of the microspheres with diameter 0.356, 0.771, 0.989, and 1.826µm were 2.09, 4.52, 5.80, and 10.72 respectively. Since microspheres were coated on the black metal slide surface after drying, the relative refractive index should be m = n<sub>polystyrene</sub>/n<sub>air</sub> = 1.6/1.0 = 1.6. The scatter intensity function  $F(\theta, \phi)$  was calculated using the software MieTab (version 8.38.02). Given the size parameter *x* and the refractive index ratio *m*, the value of the phase function

 $F(\theta, \phi)$  at each angle within the range of 0°<θ<180° can be easily obtained in MieTab as shown in Figure 10. It is important to note that unlike in transmission mode, the light is scattered directly backward in reflectance mode. Therefore, the scattering angle range for high NA is 146°<θ<180° and the scattering angle range for low NA is 170°<θ<180°. According to the settings of the microscope setup used, we can calculate OSIR by using the following equation:

$$OSIR = \frac{\int_{\phi=0}^{360^{\circ}} \int_{\theta=146^{\circ}}^{180^{\circ}} F(\theta,\phi) \sin \theta d\theta d\phi}{\int_{\phi=0}^{360^{\circ}} \int_{\theta=170^{\circ}}^{180^{\circ}} F(\theta,\phi) \sin \theta d\theta},$$

where  $\theta$  is the angle between the scatter direction and the direction of propagation of the incident light,  $\phi$  is the azimuthal angle of scatter and the scatter intensity function  $F(\theta, \phi)$  represents the intensity of scattered light defined by angles  $\theta$  and  $\phi$  [23]. The numerical integration of the scatter intensity function  $F(\theta, \phi)$  multiplying  $sin\theta$  was calculated in Matlab, where we multiplied the value of the phase function  $F(\theta, \phi)$  obtained in Mietab at each angle with its corresponding  $sin\theta$  and added them all up. Finally, the scatter ratio, OSIR, was obtained by dividing the numerical integration of the high NA scatter intensity within 146°< $\theta$ <180° by the numerical integration of the low NA intensity within 170°< $\theta$ <180°. Thus, the OSIR of microspheres with diameter 0.356, 0.771, 0.989, 1.826µm is 8.65, 7.5, 4.2, and 2.05 respectively, decreasing with increasing diameter.



**Figure 10.** Simulation of the phase function  $F(\theta,\phi)$  of microspheres with diameter 0.356 µm (blue line), 0.771 µm (red line), 0.989 µm (yellow line), and 1.826 µm (purple line) at each angle within the range of 0°< $\theta$ <180° for index ratio m=1.6 obtained from Mietab. The inset shows a magnified image of the region between 145° and 180°.

In the initial OSI setup, the OSIR was measured using the signal from forward scattered light in transmission mode to predict local particle size [23]. To show similarities and differences between forward and backscatter for the OSIR measurement, a total number of 25 different diameters ranging from 0.05-1.826µm were selected to perform the

simulations of both forward and backscattered OSIR based on the method detailed in this section, in which the corresponding values of the phase function  $F(\theta, \phi)$  were obtained in Mietab and the numerical integration and division were conducted in Matlab. For comparison, the relative refractive index for the calculation of the forward scattered OSIR was set to be the same as that in backscatter calculation, m=1.6. With the same value of the scattering angle range with 34° for high NA and 10° for low NA, the forward scattered OSIR was obtained by dividing the numerical integration of the high NA scatter intensity within 0°< $\theta$ <34° by the numerical integration of the low NA intensity within 0°< $\theta$ <10°. The calculation equation is defined as

$$OSIR = \frac{\int_{\phi=0}^{360^\circ} \int_{\theta=0}^{34^\circ} F(\theta,\phi)\sin\theta d\theta d\phi}{\int_{\phi=0}^{360^\circ} \int_{\theta=0^\circ}^{10^\circ} F(\theta,\phi)\sin\theta d\theta d\phi}.$$

All OSIR values were input into Microsoft Excel for curve fitting. Simulations of the dependence of both the forward and the backscattered OSIR on particle diameter for index ration m=1.6 are shown in Figure 11 (detailed data is shown in the Appendix). For diameter <1 $\mu$ m, we can observe that the forward scattered OSIR decreased monotonically with increasing diameter presenting a smooth curve with a downward trend. However, the backscattered OSIR showed a rising trend first between diameter 0.05 $\mu$ m and 0.2 $\mu$ m and then decreased irregularly with large fluctuations. The results indicate that it may be unlikely to predict the particle size precisely based on the measurement of the backscattered OSIR for index ratio m=1.6 owing to its nonmonotonic dependence on

sphere diameter.

Regarding the index ratio m, when angles of high-NA and low-NA were fixed, Boustany *et al.* has shown that the OSIR is independent of index ratio m, for 1<m<1.2, with m~1 for subcellular organelles [23]. To investigate how a lower index ratio m affects the backscattered OSIR measurement, the simulations of both the forward and the backscattered OSIR as function of particle diameter for index ration m=1.04 were performed and shown in Figure 12. For the whole diameter range, the forward scattered OSIR decreased monotonically with diameter. On the other hand, the backscattered OSIR for m=1.04 fluctuated even more compared with the backscattered OSIR for m=1.6. The low NA angle of collection of scattered light may be a limiting factor when using the backscattered OSIR to predict the particle size. To improve the system in the future, we can test a low NA angle lower than 10° to see if the backscattered curve would oscillate less.



**Figure 11.** Simulations of the dependence of the forward scattered and the backscattered OSIR on particle diameter ranging from 0.05-1.826µm for index ratio m=1.6. The data from the backscattered OSIR (yellow dot) is compared with the data from the forward scattered OSIR (blue dot).



**Figure 12**. Simulations of the dependence of the forward scattered and the backscattered OSIR on particle diameter ranging from 0.05-1.826µm for index ratio m=1.04. The data from the backscattered OSIR (yellow dot) is compared with the data from the forward scattered OSIR (blue dot).

#### 3.5.3 Image Capture and Analysis

The background subtracted high NA images, the background subtracted low NA images, and the OSIR images of microspheres with diameter 0.356, 0.771, 0.989, and 1.826µm are shown in Figure 13. Since the background images were subtracted from both the high and low NA images, the background subtracted images obtained a relatively clean background, where the black color represented the value 0. Note that there is still some background signal which cannot be eliminated completely due to the irregular surface of the matte black metal slides. When the scattering signal from the microspheres is pronounced, these uncompletely eliminated background signal can be ignored. It can be observed from the background subtracted images that the overall scattering intensity in the background subtracted high NA images is higher than the overall scattering intensity in the background subtracted low NA images. It is because that the collecting angle for the low NA images, 10°, is much smaller than the collecting angle for the high NA images, 34°, and provides more light blocking. In the OSIR images, the pixels directly encode the local value of OSIR, which corresponds to the intensity ratio of wide-to-narrow angle optical scatter

Furthermore, the pixel histograms presenting the distribution of OSIR values of the microspheres are shown in Figure 14. In each pixel histogram, the number of pixels indicates the distribution frequency of the backscattered OSIR value and the red vertical line serves as the reference line showing the corresponding theoretical prediction value of

the backscattered OSIR. A pixel histogram of the OSIR image roughly gives the distribution of particle sizes in the cells. In the future, one can use such size distributions to quantify changes in cell function under specific experiments conditions. Based on the Mie theory, the theoretical prediction values of the backscattered OSIR obtained in Section 3.5.2 are 8.65, 7.5, 4.2, and 2.05 for microspheres with diameter 0.356, 0.771, 0.989, 1.826µm respectively. The pixel histograms show that the maximum value of pixel number appears at around 7 in (B), 4 in (C), and 1.7 in (D), which decreases with the diameter increasing and is close to the theoretical prediction value. However, for microspheres with diameter 0.356µm in (A), a great discrepancy is found between the theoretical prediction value 8.65 and the experimental measurement of the backscattered OSIR is concentrated in the range from 1 to 4. Although the diameter of these microspheres, 0.356µm, is less than the spatial resolution of the microscope setup whose theoretical calculated value is roughly 0.6µm (detailed in Section 3.1), the resolution limitation from the microscope setup is not the main cause of this deviation. The theoretical prediction value of the OSIR for the 0.365µm microspheres is 8.65 in (A) and has already taken into account the resolution of the microscope since this OSIR value is limited by the highest NA angle (34°) achievable for the high NA measurement. In particular, when the high angle (or resolution) becomes limited, one would expect the OSIR values to start flattening out around 9.5 as shown by the left portions of the curves in Figure 11. Instead, the deviation in the OSIR value of the 0.356µm microspheres is likely caused by the tendency of the 0.356µm microspheres to

clump together after drying and therefore look like particles with larger diameter and lower OSIR. To improve the measurement, the aqueous suspension of the 0.356µm microspheres needs to be diluted to lower concentrations to prevent microspheres from clumping. It can be observed from the Image (D) that the measured OSIR value of the 1.826µm microspheres spreads in the OSIR range of 1 to 3 compared with steep changes in the measurements of the  $0.771 \mu m$  and  $0.989 \mu m$  microspheres. This is likely caused by the flattening of the OSIR response at larger sizes ranging from 1µm to 1.8µm as shown in Figure 11. Such flattening of the OSIR response may lead to an imprecise measurement of the OSIR in this specific diameter range. The spread in OSIR towards lower values may be likely caused by the clumping of the spheres. The actual variance of sphere sizes in the samples can be validated by a scanning electron microscope (SEM) in the future. The OSIR measurement of onion epidermal cells are shown in Figure 15. We can observe from these images that the edges of the onion cells are highlighted. Based on the OSIR value, each color corresponds to a size.



**Figure 13.** The background subtracted high NA images (left panel), the background subtracted low NA images (middle panel), and the backscattered OSIR images (right panel) of microspheres with diameter 0.356 $\mu$ m (A), 0.771  $\mu$ m (B), 0.989  $\mu$ m (C), and 1.826  $\mu$ m (D). Color bars in the background subtracted high NA images and the background subtracted low NA images represent the value of scattering intensity. Color bar in the OSIR images represent the backscattered OSIR images represent the backscattered OSIR images represent the backscattered OSIR value.



**Figure 14.** The pixel histograms obtained from the backscattered OSIR images of microspheres (shown in Figure 13) with diameter  $0.356\mu$ m (A),  $0.771 \mu$ m (B),  $0.989 \mu$ m (C), and  $1.826 \mu$ m (D). The red vertical line with its corresponding value in each image represents the theoretical value which was calculated based on Mie theory. The distribution data within the range of 0 to 1 was omitted.



**Figure 15.** The onion cell high NA image (left panel), the onion cell high low NA image (middle panel), and the onion cell backscattered OSIR images (right panel).

## 3.5.4 Conclusion

The simulations of the dependence of the forward scattered and the backscattered OSIR on particle diameter ranging from 0.05-1.826µm with index ratio m=1.6 show differences between the OSIR measurement using backscatter and forward scatter. In the diameter range of 0 to 1µm, the forward scattered OSIR decreased monotonically as diameter increased whereas the curve of the backscattered OSIR fluctuated. For a lower index ratio m=1.04, the backscattered OSIR fluctuated even more as a function of particle size. The actual measurements of the backscattered OSIR of microspheres with diameter 0.356, 0.771, 0.989, and 1.826µm using the OSI microscope setup were performed. The background subtracted high NA images, background subtracted low NA images, backscattered OSIR images, and pixel histograms were obtained. For microspheres with diameter 0.771, 0.989, and 1.826µm, the measured values of the backscattered OSIR agreed well with theoretical prediction. However, the measurement of microspheres with diameter 0.356µm showed a great deviation from the theoretical prediction, which may derive from the clump of the 0.356µm microspheres after drying. It can be concluded that by using non-reflective matte black metal slides to mount samples, our OSI setup can perform the measurement of the backscattered OSIR.

#### 3.6 Imaging Bovine Aortic Endothelial Cells (BAEC) in An Aqueous Medium

The ultimate goal of our OSI setup is to perform imaging cells or tissues in an aqueous

medium combining with the ZnOnano-QCM biosensor, where the matte black metal slides cannot be applied. The reflectance mode provides a convenient way for live cell imaging on the top side of the specimen without additional transmitted light, allowing the ZnOnano-QCM biosensor operating at the bottom. However, in reflectance mode, the specular reflection from the surface of the aqueous medium as well as the cell layer may affect imaging. Here, we conduct a backscattering experiment on the BAEC maintained in an aqueous medium to show the potential effect of the specular reflection and discuss how it may be blocked. The OSIR measurements of onion epidermal cells were also successfully performed. The edges of the onion cells were highlighted in the onion cell OSIR image.

#### 3.6.1 Materials and Methods

Bovine Aortic Endothelial Cells (BAEC, Clonetics Lonza, Chicago, IL) were cultured by Mohammad Naser who is a Ph.D. student researcher of the Bio-Optics Laboratory in the department of Biomedical Engineering at Rutgers. For optical viewing, Mohammad Naser also helped me grow the cells on no. 1 glass coverslips (Fisher Scientific, Pittsburgh, PA). A specially fabricated steel plate was used to mount the coverslips with live cells as illustrated in Figure 16. Such steel plate was fabricated with a 1cm circular hole drilled through the center. The coverslip with live cells facing the inside of the chamber was first mounted on the bottom side of the hole with vacuum grease. The edge of the coverslip was fixed by a layer of VaLaP (a 1:1:1 mixture of vaseline, lanolin, and paraffin) wax creating a watertight and rigid seal. A soldering iron on the low setting (~15 W) was used to melt the VaLaP wax and dispense molten VaLaP wax to the coverslip edge. Finally, the plate was turned over and the hollow chamber formed by the wall of the through hole and the coverslip was filled with viewing medium (CO<sub>2</sub>- independent Leibovitz L-15 medium (Invitrogen) supplemented with 10% FBS at room temperature and room air) by pipette. Immediately upon completing the cell mounting, the steel plate was carefully moved and mounted on the sample mount of the OSI microscope setup for imaging without a lid on the top side of the hole. The dark-field filter was used to generate dark-field images by blocking the specular reflection from the surface of the aqueous medium. When imaging, the setup should stay still to prevent the medium surface from fluctuating. The method of imaging was the same as described in Section 3.3.



**Figure 16.** The steel plate mounted with the cells for imaging. The steel plate is fabricated with a circular centered through hole with diameter 1cm. The coverslip with live cells facing the inside of the chamber is mounted on the bottom (Underside View) with vacuum grease. The edge of the coverslip is applied by a layer of VaLaP wax. The hollow chamber is filled with viewing medium (Side View).

#### 3.6.2 Image Capture and Analysis

Figure 17 shows the dark-field and bright field image of the BAEC in an aqueous medium. While the BAEC were resolved in the bright field image, we failed to obtain clear dark-field BAEC images. In (B) the dark-field image shows that there is a significant amount of light from specular reflection leaking and the BAEC are not resolved clearly. It illustrates that the specular reflection from the surface of the aqueous medium cannot be perfectly blocked by the dark-field filter we fabricated. When the cells are in an aqueous medium, there are two specular reflections: one from the cell layer and another from the top of the fluid. The specular reflection from the cell layer can be blocked more effectively and perhaps be avoided if the cells are grown on a non-reflective black surface. However, the specular reflection from the top of the liquid may not focus precisely at the center of the Fourier plane since the liquid can move, making it difficult to be blocked by a dark-field filter.

To solve this problem, we may be able to immerse the lens which collects the backscattering light into the aqueous medium with the cells, thereby increasing the numerical aperture of the lens and removing the specular reflections from both the surface of the aqueous medium and the cell layer. A more collimated light source can allow scattered light to focus more precisely and may also improve the image quality.



**Figure 17.** Images of the BAEC taken by the microscope setup using green-light illumination (535nm). (A) Imaging in bright field without any filtering. (B) Imaging in dark field with a center stop at the rear focal plane of L3.

## 3.6.3 Conclusion

The glass coverslips with the live BAEC was mounted on the special steel plate, where the BAEC were maintained in the aqueous medium. When performing dark-field imaging with the dark-field filter, we failed to obtain clear dark-field images because the specular reflection could not be blocked perfectly. A lot of improvements remain be made in the future to remove the specular reflection.

## Chapter 4

# **Conclusion and Future Work**

In conclusion, in this thesis we designed and constructed a miniaturized OSI microscope setup adopting reflectance mode with Fourier filtering based on the original OSI setup. As an optical imaging modality, the OSI microscope system is expected to combine with the impedance measurements made by the ZnOnano-QCM biosensor. The specifications and performance of this OSI microscope setup were measured and evaluated theoretically and experimentally. We validated that the microscope aperture of our setup was not cut off and had an NA of 0.55, the true magnification was 8X, and the field of view was 596 x 446µm, which all reached the design requirements. On the other hand, spatial resolution was calculated as 0.6µm theoretically, and can be precisely measured using a standard resolution target or by obtaining a PSF with a 0.3µm microsphere image in the future. To get images at the resolution capability of the microscope, the current aspheric lens system can be adjusted to obtain a larger magnification by substituting L2 or L4 with an aspheric lens with a longer focal length.

Using a dark-field filter consisting of a central plastic "dot" affixed to a coverslip placed in a conjugate Fourier plane, we tried to acquire dark-field images of the Ted Pella stage micrometer and the BAEC maintained in saline on a glass coverslip but were unsuccessful because the specular reflection from the sample surface could not be blocked perfectly by the Fourier filter. To remove the specular reflection in the future, we can substitute the green LED light source we used with a more sophisticated light source like a fiber-coupled LED. Fiber-coupled LEDs can greatly improve the collimation of the transmitted light and are more ideal to be placed within a standard humidified incubator for their tiny size. Regarding the dark-field filter, we should improve workmanship to fabricate dark-field filters with a circular shape preventing light leakage. For example. instead of using a blade to cut down the edges, we can employ the microfabrication technique to make a well-controlled dark-field filter out of metal of any size and shape we need. We can also try to immerse the lens which collects scattering light into the aqueous medium with the cells, which increases the numerical aperture of the lens and removes the specular reflection by avoiding the effects of the refractive index mismatch at the sample surface.

Apart from using a dark-field filter, we also tried to acquire dark-field images using nonreflective matte black metal slides for sample mount. Onion epidermal cells and microspheres were imaged. The theoretical simulations of the dependence of the forward scattered and the backscattered OSIR on particle diameter ranging from 0.05-1.826µm indicate that there are differences between the OSIR measurement using backscatter and forward scatter. Namely, the backscatter OSIR fluctuates more as a function of particle size compared with the forward scatter OSIR which varies monotonically as a function of sphere diameter. For a lower index ratio m=1.04, the backscattered OSIR fluctuated even more as a function of particle size. To improve the system in the future, we can test a low

NA angle lower than 10° to see if the backscattered curve would oscillate less. Using circular high- and low- NA filters, we successfully measured OSIR values of microspheres with diameter 0.356, 0.771, and 0.989µm but the measurement of microspheres with diameter 0.356µm showed a great deviation from its theoretical prediction. The results demonstrate that the non-reflective matte black metal slides provide another way for the setup to acquire dark-field image and allow the backscattered OSIR measurement. However, the matte black metal slides cannot be used to mount live mammalian cells maintained in aqueous media. This means that this method is not suitable for investigations on animal tissues or cells but it indicates that using non-reflective black cell culture dishes may greatly improve optical scattering measurement when adopting reflectance mode because of low background signal. The OSIR measurements of onion epidermal cells were also successfully performed. The edges of the onion cells were highlighted in the onion cell OSIR image. In the future, one can use such size-related quantitative information to quantify changes in cell function.

The OSI microscope system that was developed provides a proof-of-principle for conducting the OSIR measurement in reflectance mode. A number of refinements, such as high NA collection lens, well-collimated illuminating light source and rounded dark-field filter, should be conducted to improve the setup's performance and to eliminate the background due to specular reflection in samples in the future. In addition to high- and low-NA filtering, various Fourier filters can also be designed for the OSI microscope to
select different scattering parameters so that this OSI microscope setup can be further extended to other applications. For example, optical Gabor-like filtering is very useful to gain information of dimension and shape of unknown particles. In the previous work, optical Gabor-like filtering with a digital micromirror device successfully quantified the orientation and geometrical aspect ratio of subcellular organelles [24]. In the future, we seek to apply this setup to combine with the ZnOnano-QCM biosensor for monitoring cellular dynamic morphological changes in biological study without extrinsic label material staining.

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## Appendix

Diameter(µm)	Forward scattered OSIR	Backscattered OSIR
0.05	9.72	9.77
0.10	9.65	9.84
0.15	9.54	9.97
0.20	9.40	10.20
0.25	9.21	10.60
0.30	9.00	8.12
0.36	8.52	8.65
0.40	7.96	9.15
0.45	7.62	8.41
0.50	7.20	8.41
0.55	6.28	8.11
0.60	6.02	8.25
0.65	5.55	7.22
0.70	4.52	6.18
0.77	4.20	7.50
0.85	3.11	4.79
0.90	2.92	7.10
0.95	2.53	4.09
0.99	2.48	4.20
1.05	2.28	4.11
1.10	3.05	4.40
1.20	3.31	2.46
1.30	2.29	2.91
1.50	1.90	3.13
1.83	2.20	2.05

 Table 1.
 Detailed forward and backscattered OSIR data of spheres with different diameters.