Optical reflectance contrast of micro- and nano-structures revealed with intravital micro-OCT

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**Optical Reflectance Contrast of Micro- and Nano-
structures Revealed with Intravital Micro-OCT**

CHEN SI

SCHOOL OF ELECTRICAL & ELECTRONIC ENGINEERING

2019

Optical Reflectance Contrast of Micro- and Nano-structures Revealed with Intravital Micro-OCT

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School of Electrical & Electronic Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

8 Nov 2019
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Nov 08, 2019

Liu Linbo
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Liu Linbo

Authorship Attribution Statement

This thesis contains material from 3 papers published in the following peer-reviewed journals where I was the first author.

Chapter 4 is published as S. Chen, X. Liu, N. Wang, Q. Ding, X. Wang, X. Ge, E. Bo, X. Yu, H. Yu, C. Xu, and L. Liu. Contrast of nuclei in stratified squamous epithelium in optical coherence tomography images at 800 nm. *J Biophotonics* (2019). DOI: 10.1002/jbio.201900073.

The contributions of the co-authors are as follows:

- A/Prof Liu provided the initial project direction, supervised the study and co-edited the manuscript drafts with Asst Prof C. Xu.
- I co-developed the optical system with Dr X. Liu, and N. Wang;
- I designed the study and performed all the sample preparation and image acquisition work, analyzed the data, conducted image interpretation and wrote the manuscript draft.
- Dr X. Liu, and Mr X. Wang developed image acquisition, processing, and display software, as well as the dispersion correction algorithm.
- Dr E. Bo, X. Yu, and X. Ge assisted with the animal experiments.
- I conducted all histology and microscopy with assistance from Dr Q. Ding.
- Dr Q. Ding and Prof H. Yu provided the support of human esophageal tissues.
- All the authors edited the manuscript draft critically.

Chapter 5 is published as S. Chen, X. Ge, X. Liu, Q. Ding, N. Wang, X. Wang, S. Chen, H. Liang, Y. Deng, Q. Xiong, G. Ni, E. Bo, C. Xu, H. Yu, and L. Liu. Understanding optical reflectance contrast for real-time characterization of epithelial precursor lesions. *Bioengineering & Translational Medicine* (2019). DOI: 10.1002/btm2.10137.

The contributions of the co-authors are as follows:

- A/Prof Liu provided the initial project direction and co-supervised the study with Prof H. Yu and Asst Prof C. Xu.
- I designed all the studies and performed all the image interpretation work, and wrote the manuscript draft.
- Dr X. Ge and I co-conducted the simulation work.
- I co-developed the optical system with Dr X. Liu, and N. Wang;
- Dr Q. Ding and Prof H. Yu provided the support of human gastrointestinal tissues and Mr Y. Deng performed OCT imaging of these tissues at Renmin Hospital of Wuhan University.
- Dr X. Liu and Mr X. Wang, developed image acquisition, processing, and display software, as well as the dispersion correction algorithm.
- I developed animal models and conducted imaging experiments with assistance of Ms. S. Chen and Mr. H. Liang.
- Dr E. Bo, X. Ge, G. Ni and Mr Q. Xiong assisted with the animal experiments.
- Mr. H. Liang, Ms. S. Chen and I conducted all histological analysis including tissue sectioning and staining.
- All authors edited the manuscript draft critically.

Chapter 6 is published as S. Chen, X. Liu, N. Wang, X. Wang, E. Bo, X. Yu, S. Chen, and L. Liu. Visualizing micro-anatomical structures of the posterior cornea with micro-optical coherence tomography. *Scientific Reports* (2017). DOI: 10.1038/s41598-017-11380-0.

The contributions of the co-authors are as follows:

- A/Prof Liu provided the initial project direction and edited the manuscript drafts.
- I designed the study, analyzed the data, conducted image interpretation and wrote the manuscript draft.
- I co-developed the optical system with Dr X. Liu, and N. Wang;
- I performed all the image acquisition work with assistance from Dr X. Liu, N. Wang, E. Bo and X. Yu.

- Dr X. Liu and Mr X. Wang developed image acquisition, processing, and display software, as well as the dispersion correction algorithm.
- Ms S. Chen assisted with sample collection from local slaughter house.
- All the authors edited the manuscript draft critically.

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Summary

Optical coherence tomography (OCT) provides depth-resolved three-dimensional images allowing the visualization of subsurface microstructures over large areas *in situ* and thus is a promising tool for epithelial disease screening. However, it has generally been relegated to a nonspecific diagnostic tool because the optical reflectance (back-scattering) contrast in OCT images is poorly understood although some contrast agents are available for OCT. This thesis focuses on elucidating the microstructural and ultrastructural bases underlying optical contrast to explore if OCT could provide diagnostic information in epithelial tissues with specificity. We investigate the back-scattered intensity of different intracellular scatterers at the micrometer and nanometer scale using finite-difference-time-domain method numerically and cellular-resolution OCT (μ OCT) experimentally. We uncover that there exist correlations between reflectance contrasts (optical reflectance intensity differences among structures) and intracellular scatterers, and clarify that the nuclei in the nonkeratinized stratified squamous epithelium are low-scattering in the core and high-scattering at the nucleocytoplasmic boundary while the scattering properties of cytoplasm is cell-type specific depending on its ultrastructural features. We further validate that these new understandings on reflectance contrast enable the characterization of precancerous lesions in OCT images with specificity, which may have a significant impact on the screening and surveillance of epithelial cancers.

In addition, we also make an effort to optimize OCT imaging system by optimizing the spectral shape of the detected signal. We design a novel cost-effective interferometer-in-spectrometer scheme where the reference beam is spectrally shaped when passing through the zero-order diffraction channel of the grating prior to interfering with the sample light at the spectrometer input fiber tip. Therefore, the spectral modulations caused by the grating and the camera are compensated for by improvements in axial resolution by 10% and ranging depth by 25% without impairing the system sensitivity.

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Acronyms

AB	Alcian Blue
AVOVA	Analysis of Variance
BS	Beam Splitter
CCD	Charge-Coupled Device
DM	Descemet Membrane
DOF	Depth of Focus
FD-OCT	Fourier Domain Optical Coherence Tomography
FDTD	Finite-Difference Time-Domain
FF-OCT	Full Field Optical Coherence Tomography
FOV	Field of View
FWHM	Full Width at Half Maximum
HE	Hematoxylin-Eosin
IM	Intestinal Metaplasia
IVCM	In Vivo Confocal Microscopy
NA	Numerical Aperture
OCM	Optical Coherence Microscopy
OCT	Optical Coherence Tomography
PAS	Periodic-Acid Schiff
PSF	Point-Spread Function
SCE	Simple Columnar Epithelium
SD-OCT	Spectral Domain Optical Coherence Tomography
SMF	Single Mode Fiber
SNR	Signal-to-Noise Ratio
SSE	Stratified Squamous Epithelium
TD-OCT	Time Domain Optical Coherence Tomography
μOCT	Micro Optical Coherence Tomography

Chapter 1

Introduction

1.1 Motivation

Epithelial cancers such as esophageal cancer, gastric cancer, and colorectal cancer rank in the leading causes of mortality worldwide¹⁻⁴. Detecting cancers at the early or precancerous stages is challenging as their early-stage lesions are difficult to be recognized by the naked eyes and can be focally and heterogeneously distributed over large mucosal areas. Biopsies often have to be randomly performed on multiple locations of the mucosa, with the hope of sampling changes of particular clinical relevance, which unfortunately is frequently missed^{2,3,5}. In routine clinical practice, subsequent histological analysis of stained, thin sections from biopsied specimens is conducted for a definite diagnosis, where specimens are sliced to thin sections (3-5 μm) and stained with contrast agents. With clear understandings on the underlying mechanistic chemistry of staining, structures of interest can be specifically highlighted. Clearly, the whole workup is time-consuming and labor-intensive which limits the capability of clinicians to immediately characterize the lesions, possibly leading to unnecessary biopsies or the need for repeated biopsies. In addition, concerns on the high costs from histology have been raised when lesions of limited clinical importance are increasingly found in screening as is the case in diminutive colorectal polyps^{6,7}.

To make things worse, in organs such as ocular cornea, invasive biopsy is hazardous and impossible to be performed in living organisms. Globally, corneal disease ranks as the 2nd leading cause of visual impairment and the 4th leading cause of blindness^{8,9}. Corneal transplantation is the major strategy for visual restoration where approximately 50% transplantation is conducted to treat corneal endothelial dystrophy⁹. With the increasing improvement of surgical devices and skills, surgeons have making it possible to transplant thin graft which contains only the posterior layers of cornea and thus dramatically decrease postoperative complications in full-thickness corneal

transplantation^{10,11}. Effective evaluation of the status of donor endothelial cell which is the critical factor determining corneal graft survival¹² as well as accurate delineation of the corneal allograft interface would be of great significances in pre-, intra-, and post-operative evaluation.

Optical coherence tomography (OCT), a representative optical imaging modality, has attracted significant interests for ‘virtual histology’, where ‘histology-like’ subsurface microstructures could be resolved *in situ* without the need of resection and subsequent histology. Particularly with the development of high-speed Fourier-domain OCT (FD-OCT)¹³⁻¹⁶, it has been demonstrated to be able to image microstructures across large mucosal areas, making it a promising tool for epithelial cancer screening. As we all know, in optical imaging like OCT imaging, the reflectance (back-scattering) contrast is provided by the refractive index differences between microstructures. Whereas, unlike histological methods where the image contrasts have been fully understood and well matched with the physiological events in tissues, optical-reflectance image interpretation has ever relied on morphological or architectural similarities to histology, leaving the biological bases underlying the contrast poorly understood^{14,15,17-19}. This knowledge gap has relegated OCT modalities to nonspecific morphometric tools where reflectance signals that may carry diagnostically important information are missed or misinterpreted, precluding their applications in a broader research or clinical arena.

It is important to note that visualizing tissue structure and/or molecular expression are the predominant methods of diagnosing tissues. In this thesis, we suggest that there might be additional information in knowing how reflectance intensities correlate with various biological states. In other words, in addition to being able to visualize different structures via reflectance contrast (e.g. nuclei and epithelial layers), we proposes that quantifying changes in that contrast (e.g. bright becoming dark), can also be informative and indicative of biological changes that are of diagnostic value.

1.2 Objectives

The main objective of the thesis work is to explore the specificity of OCT signals by elucidating the ultrastructural bases underlying optical reflectance (back-scattering) contrast, with the follow 3 specific aims:

- 1) To numerically analyze the scattering features of nuclear and cytoplasmic inclusions by conducting finite-difference time-domain (FDTD) simulations;
- 2) To design, develop, and optimize a subcellular-resolution SD-OCT system for imaging epithelial tissues from model animals and human tissues *ex vivo* or *in vivo* so that the theoretical FDTD results could be verified with experimental evidences.
- 3) To establish OCT as a subcellular-level imaging tool for the screening of precancerous lesions by validating against histological and pathological analysis of imaged tissues.

1.3 Major Contributions of the Thesis

This thesis work focuses on disclosing the correlation between optical reflectance contrasts and the ultrastructural features of epithelial cells in intact tissues. The improved understanding on OCT images, which is a fundamental step to realize the potential of an imaging modality, offers us the possibilities to characterize epithelial cells with specificity based on the reflectance contrast and will consequently help with real-time characterization of epithelial diseases including precancerous lesions. In this thesis, I take efforts to interpret cellular-level morphological changes that are seen with reflectance contrast to deduce the physiological states of tissues.

First of all, we unify the knowledges on the scattering characteristics of the cell nucleus in mammalian epithelial tissues which is a diagnostically important subcellular structure as nuclear atypia is closely correlated with tumorigenesis. In contrast to previous studies where epithelial cell nuclei are reported to be either high scattering (or ‘bright’) or low scattering (or ‘dark’), we for the first time clarify that the nuclei are constantly low scattering in the core; those previously reported high-scattering nuclear signals are actually from the nucleocytoplasmic boundary, and the low-scattering nuclear cores are missed possibly owing to the insufficient axial resolutions.

Second, we uncover that the scattering characteristics of cytoplasm attributed by the micro- or nano-structural scatterers within the cell cytoplasm could be cell-type specific. Different from previous studies which exclusively focus on assessing the scattering contributions from micrometer-scale spherical scatterers, we evaluate the back-

scattering properties of micrometer- or nanometer-scale scatterers including spherical mucin granules, cylindrical keratin filaments and microvilli both numerically and experimentally over a wide variety of epithelial tissues. More importantly, we for the first time establish that above-mentioned intracellular scatterers present varied back-scattered intensity under different clustering or packing states. As a result, we are able to differentiate those ‘immature’ or ‘ectopic’ cells in epithelial tissues based on the differential scattering profiles among cells. These improved understandings on the scattering profiles of epithelial cells enable the characterization of precancerous lesions, which complements the current morphology- or architecture- based diagnostic criteria in the effort to realize the goal of ‘virtual histology’, and would ultimately have a significant impact for the screening of epithelial diseases including cancers.

As endothelial cells are specialized epithelial cells that are spatially organized to a monolayer lining the internal surface of an organ, we also exert efforts to optimize our understandings on the back-scattering features of the endothelial cells in transparent cornea (i.e. endothelium) as well as other cellular and extracellular components in the posterior cornea with μ OCT. We for the first time disclose that the polygonal endothelial cells have a flat apical side but a ‘dome-shaped’ basolateral side in the cross-sectional plane and monitor their longitudinal variations in the scattering intensity during cell degeneration. In accordance with previous observations, we also recognize that the keratinocytes and extracellular components such as collagen and nerve fibers in corneal stroma are high scattering. The discoveries of these subcellular-level components with μ OCT not only enrich our knowledges on corneal microarchitectures but also help with the pre- or post- operative evaluation of corneal status and graft survival in corneal transplantation.

1.4 Organization of the Thesis

The following chapters are included in this thesis:

Chapter 2 serves as the theoretical foundations of this thesis work. Firstly, a general introduction of the working principle is given and key parameters of an OCT imaging system are introduced. Thereafter, we focus on illustrating spectral-domain OCT (SD-OCT) related theories including Fourier-domain low-coherence interferometry,

sensitivity fall-off and signal-to-noise ratio. Finally, the OCT image formation theories are also illustrated.

Chapter 3 describes the development of two SD-OCT systems, which are capable of imaging subcellular and extracellular structures within biological tissues. We firstly introduce the development of a subcellular-resolution SD-OCT (i.e. μ OCT) system with a compact, flexible sample-arm probe to facilitate image acquisition *in vivo* and test its performance in axial and lateral resolution, and ranging depth. Given the sub-optimal spectral responses of the spectrometer components which sacrifice axial resolution and ranging depth, we then report a novel SD-OCT design which integrates the interferometer into the spectrometer, termed interferometer-in-spectrometer (IIS) scheme. This IIS scheme is to shape the detected spectrum in the reference arm to improve axial resolution and ranging depth without compromising the system efficiency. System performances in axial resolution, ranging depth and imaging capability of the IIS scheme are experimentally compared with those of a standard scheme.

Chapter 4 and Chapter 5 elucidate the micro and nano-scale ultrastructural bases underlying optical reflectance contrast and clarify the scattering characteristics of the cell nuclei (Chapter 4) and several micrometer- or nanometer-scale inclusions in the cytoplasm (Chapter 5) using different types of epithelial tissues from mammals including human both *ex vivo* and *in vivo*. In each chapter, we attempt to evaluate the scattering characteristics of intracellular components numerically using the FDTD method and then validate the simulation results experimentally in normal epithelial tissues using the subcellular-resolution OCT (i.e. μ OCT). We further demonstrate that the improved understandings of OCT image signals enable us to characterize precancerous lesions and early-stage epithelial cancers in real-time and *in situ* using human endoscopically resected specimens and specimens from small animals.

Chapter 6 demonstrates the scattering characteristics of the monolayer epithelium (endothelium) and cellular and extracellular structures in transparent corneal tissues rather than scattering tissues as discussed in Chapter 4 and 5. The back-scattering intensity of corneal layers and its cellular components including endothelial cells, keratinocytes, collagen fibers and nerve fibers are observed in small and large animals using the subcellular-resolution SD-OCT (i.e. μ OCT).

Chapter 7 summarizes the thesis works and their potential impacts, and proposes research directions for future work.

Chapter 2

Optical Coherence Tomography

Theory

2.1 Introduction to OCT

Optical coherence tomography (OCT) has been established as an important optical imaging modality in biomedical research and clinical practice particularly in the field of ophthalmology. It provides high-resolution, depth-resolved, cross-sectional images in real time by measuring the echoes of backscattered light from biological tissue without the need of excision and tissue processing as in histopathology^{20,21}. These features make it a powerful tool in clinical situations where invasive biopsy is hazardous or even impossible (e.g. diseases in eyes, arteries, or nerves), where biopsy sampling error is common but missed diagnosis is life-threatening (e.g. gastrointestinal precancerous lesions), and where longitudinal observations is needed to trace the natural history of diseases and to evaluate treatment responses (e.g. retinal neovascularization, or corneal transplantation).

OCT works based on low-coherence interferometry which is used to measure optical echo delay of the light (**Figure 2. 1**). In a typical Michelson interferometer configuration (**Figure 2. 1a**), the incident light source of an OCT system is split by a beam splitter into a reference beam (reference arm) and a measurement beam (sample arm); the backscattered light from both the reference and sample arms is recombined by the same beam splitter and the interference signal intensity is then measured by a photodetector at the detector arm. Different from a classical laser interferometry which uses a monochromatic light source (narrow linewidth), OCT uses polychromatic light source (broad bandwidth) so that optical echoes from different depths of the sample could be differentiated (**Figure 2. 1b**). Specifically, if a nonochromatic (or narrow linewidth)

light source is used, the coherence length is long and interference signal would be observed over a wide range of distance, so that different layers along the depth are not distinguishable (**Figure 2. 1b**; left); therefore, OCT always uses polychromatic (or broad bandwidth) light source, so that the coherence length is short and interference signal will be observed only within the coherence length, achieving thin axial optical sectioning (**Figure 2. 1b**; right).

Figure 2. 1 Working principles of OCT. (a) Schematic of a Michelson interferometer in OCT imaging. (b) Narrow bandwidth light source has long coherence length (left); broad bandwidth light source has short coherence length (right). (c) Generation of axial scan (left), cross-sectional image (middle) and three-dimensional volumetric data set (right). (This figure is reproduced from reference²¹).

Interference signals from different back-scattered surfaces along the depth contribute to an axial-scan or A-scan (**Figure 2. 1c**; left). By scanning the incident optical beam transversely and performing sequential analyses of A-scans, a two-dimensional, cross-sectional image could be achieved (**Figure 2. 1c**; middle). If the incident optical beam is scanned in a two-dimensional pattern, sequential cross-sectional images (B-scans) are

generated and a three-dimensional volumetric dataset could be acquired by reconstruction (**Figure 2. 1c**; right).

2.2 Key Parameters of OCT System

2.2.1 Lateral resolution, DOF, axial and lateral FOV

As one of optical microscopic techniques, the lateral resolution of an OCT system is determined by diffraction-limited spot size of the focused optical beam. The parameters characterizing these diffraction-limited quantities are summarized in **Figure 2. 2**.

Figure 2. 2 Schematic of confocal parameters in OCT sample arm optics.

The objective lens has a focal length of f ; NA represents the effective numerical aperture of the focusing optics at the sample arm; λ is the center wavelength of the wavelength spectrum of the light source. $\omega(z)$ is the radius when the intensity dropped to $1/e^2$ of its on-axis value; ω_0 is beam waist, the radius of the beam at the focus; z_R denotes Rayleigh range which is the location where the radius of the curvature of wavefront is minimum and is the distance from the waist along the beam when the radius of the beam is $\sqrt{2}$ time as it is at the focus (i.e. $\omega(z_R) = \sqrt{2}\omega_0$).

OCT uses light sources with Gaussian-shaped spectrum. The field distribution of the Gaussian beam along the propagation direction (z direction) could be expressed as

$$E = \frac{c}{\omega(z)} \exp \left[-\frac{r^2}{\omega^2(z)} \right] \exp \left[-i \left[k \left(z + \frac{r^2}{2R(z)} \right) + \phi(z) \right] \right] \quad (1)$$

Here, c is a constant, r is the radical distance from the center axis, $R(z)$ is the radius of the curvature of the beam's wavefront, $\phi(z)$ is the Gouy phase, $\omega(z) = \omega_0 \sqrt{1 + \left(\frac{\lambda z}{\pi \omega_0^2} \right)^2}$ is the radius corresponding to the distance between the beam axis and the "edge" which is defined as when the amplitude falls to $1/e$ of its on-axis value (or intensity drops to $1/e^2$ of its on-axis value). ω_0 is the radius of the beam at the focus ($z = 0$) which is termed as the "beam waist". The value of ω_0 could be achieved by deducing the asymptote of the beam edge so that $NA \approx \tan \alpha = \lim_{z \rightarrow \infty} \frac{d\omega(z)}{dz}$ and thus $\omega_0 \approx \frac{\lambda}{\pi NA}$. Here, λ is the center wavelength of the light source; NA is the numerical aperture of the objective lens or, correctly, the effective numerical aperture of the focusing optics in the sample arm.

For each z position, the lateral point spread function (PSF) could be expressed using general Gaussian function $f(x) = a \cdot e^{-\frac{(x-b)^2}{2c^2}}$ with full width at half maximum (FWHM) $= 2\sqrt{2 \ln 2} c$. Given the width at the $1/e^2$ of the peak power of the PSF being $2\omega_0$ at the focus, the lateral resolution which is defined as the FWHM power of the lateral PSF at the focus position could be achieved:

$$\Delta x = \sqrt{2 \ln 2} \omega_0 = 0.37 \frac{\lambda}{NA} \quad (2)$$

The expression of $z_R = \frac{\pi}{\lambda} \omega_0^2$ is derived according to $\omega(z_R) = \sqrt{2} \omega_0$ and we define $2z_R$ as the depth of field b :

$$b = 2z_R = 2 \frac{\pi}{\lambda} \omega_0^2 = \frac{2\lambda}{\pi NA^2} \quad (3)$$

As revealed by **Eq. (2)**, finer lateral resolution could be achieved by using an objective lens with higher NA. Whereas, since NA also determines the depth of field b as shown

in **Eq. (3)**, a high NA will increase the lateral resolution but decrease the depth of field (or depth of focus) resulting in a trade-off between these two parameters. Clearly illustrated in **Figure 2. 3**, using a high NA objective lens could achieve fine lateral resolution but at the expense of depth of focus.

Figure 2. 3 Lateral resolution and depth of focus of OCT. The lateral resolution is determined by the standard Gaussian beam optics at the focus position and is measured at the FWHM power of the lateral PSF. NA is the numerical aperture of the optics, ω_0 is the beam waist, z_R is the Rayleigh range, b is depth of field, and Δl_c determined by the light source is the coherence length measured at the FWHM power of the axial PSF for each reflector and is termed as the axial resolution of an OCT system. (This figure is reproduced from reference²¹).

The axial field of view (FOV_{axial}) of an OCT system is defined as the FWHM power of the confocal axial response function that the intensity drops to one-half of the peak value at the focus (**Figure 2. 2**):

$$FOV_{axial} = \frac{0.221}{\sin^2\left(\frac{\alpha}{2}\right)} = \frac{0.221\lambda}{\sin^2\left(\frac{\sin^{-1}(NA)}{2}\right)} \quad (4)$$

The lateral field of view ($FOV_{lateral}$) depends on the lateral scanning system. If the input sample arm beam is rotated by the scanning components i.e. galvo scanner to a maximum one-side scan angle θ_{max} in a lateral dimension as illustrated in **Figure 2. 2**, the lateral field of view is given by

$$FOV_{lateral} = 2 \cdot f \cdot \theta_{max} \quad (5)$$

2.2.2 Axial resolution

In contrast to traditional optical microscopy, the axial resolution of the OCT system is independent of diffraction-limited spot size of the focused optical beam but is determined by the coherence length of the light source.

Figure 2. 4 Fourier transform of Gaussian-shaped “coherence function” (left) and light source spectrum (right). I_c defines the coherence length at the FWHM value and Δk is the half-width of the spectral bandwidth at $1/e$ of its maximum, F represents Fourier transform operation. (This figure is reproduced from reference²¹).

OCT normally uses Gaussian-shaped spectrum light source with low-coherence length to perform “optical sectioning”. We here assume the light source has a center wavelength of λ and a wavelength bandwidth at the FWHM of its wavelength spectrum of $\Delta\lambda$. As illustrated in **Figure 2. 4**, the corresponding Gaussian function of spectrum $S(k)$ and its Fourier transform “coherence function” $\gamma(z)$ is given by

$$\gamma(z) = e^{-z^2 \Delta k^2} \xleftrightarrow{F} S(k) = \frac{1}{\Delta k \sqrt{\pi}} e^{-\frac{(k-k_0)^2}{\Delta k^2}} \quad (6)$$

Here, F represents Fourier transform operation. The coherence length at the FWHM $l_c = \frac{2\sqrt{\ln 2}}{\Delta k}$ could be achieved from the “coherence function” $\gamma(z)$. Note $\Delta\lambda$ represents wavelength bandwidth at the FWHM of the wavelength spectrum but Δk is defined as the half-width of the spectral bandwidth at $1/e$ of its maximum here, therefore, $\Delta k = \frac{\pi}{\sqrt{\ln 2}} \frac{\Delta\lambda}{\lambda^2}$ according to the Gaussian-shaped spectrum $S(k)$. The axial resolution depend as

the coherence length at the FWHM power of the axial PSF could therefore be expressed as

$$\Delta z = \frac{2 \ln 2}{\pi} \frac{\lambda^2}{\Delta \lambda} \quad (7)$$

Where λ is the center wavelength of the light source, and $\Delta \lambda$ is the FWHM bandwidth of the power spectrum. Clearly shown in **Figure 2. 5**, higher axial resolution could be achieved using light source with shorter center wavelength and broader bandwidth light.

Figure 2. 5 Axial resolution in OCT imaging. Axial resolution versus light source bandwidth centered at 800 nm, 1060 nm, and 1300 nm. (This figure is reproduced from reference²¹).

2.3 Spectral-Domain OCT

2.3.1 Working Principle of SD-OCT

Over the past decades, OCT has evolved from time-domain OCT (TD-OCT) to Fourier domain OCT (FD-OCT) which could be subdivided into spectral domain OCT (SD-OCT) and swept source OCT (SS-OCT) with advances in imaging speed and sensitivity.

Different from TD-OCT which uses an interferometer with a scanning reference arm, time-efficient SD-OCT uses an interferometer with a fixed reference arm whose position is approximately corresponding to the sample arm (**Figure 2. 6a**)^{20,22-24}. The spectral interference fringes are detected either in parallel (SD-OCT) or in a time-lapse manner (SS-OCT) whose frequency is proportional to the optical path delay between the

reference reflector and the sample reflector (**Figure 2. 6b**). In other words, different optical echo delays (path length differences ΔL) will produce different frequency modulations (**Figure 2. 6b**) and, by Fourier transforming the spectral interference spectrum signal, the echo delays could be measured (**Figure 2. 6c**). In the detection arm, a spectrometer is used to disperse the spectral interference pattern, so that the interference spectrum could be detected by an array detector such as a charge-coupled detector (CCD) as a function of wavelength^{20,22,23}. Rescaling the spectrometer output from wavelength to frequency ($k = \frac{2\pi}{\lambda}$) and then using inverse Fourier transform, an axial-scan that includes the entire depth-resolve microstructures of the sample could be recovered. Briefly, SD-OCT measures the cross-spectral density by means of a spectrometer located at the output of the detection arm of the interferometer but without the need of mechanical modulation of the reference arm path-length as in TD-OCT, which therefore allows for faster image acquisition rate.

Figure 2. 6 Spectral domain OCT. (a) SD-OCT uses an interferometer with a broadband light source and a fixed reference arm. A spectrometer is used to detect interference pattern between light back-reflected from the reference mirror and different depths of the sample. (b) Light from the two arms have an optical echo delay and different delays result in differences in the frequency modulation. (c) Fourier transforming the interference spectrum yields to the depth information of an axial-scan. (This figure is reproduced from reference²¹).

2.3.2 Theory of Spectral Low Coherence Interferometry

In OCT, the interferometer could be illuminated by a polychromatic (broad bandwidth)

plane wave whose electric field could be expressed as $E_0 = s(k, \omega)e^{i(kz - \omega t)}$, where $s(k, \omega)$ is the electric field amplitude as a function of k and ω . Here, $k = \frac{2\pi}{\lambda}$ and $\omega = \frac{2\pi}{T}$ is the spatial and temporal angular frequency of each spectral component of the electric field, respectively.

Figure 2. 7 Schematic of a low-coherence interferometer used in SD-OCT.

As presented in **Figure 2. 7**, we assume that the beam splitter here have a wavelength-independent power splitting ratio of 0.5 (50/50). The distance between the beam splitter and the reference reflector is defined as z_R . Assuming the reference path is in air and the reference reflector have an electric field reflectivity of r_R , the field of the light incident on the beam splitter after backreflected by the reference mirror is given by $E_R = \frac{E_0}{\sqrt{2}} r_R e^{i2kz_R}$.

Divided by the beam splitter, the other part of the incidence light propagates to the sample. For an illustrative example, we assume that there are a series of discrete reflectors along the depth of the sample, with each reflector having an electric field reflectivity of $r_{S1}, r_{S2}, r_{S3} \dots$ and optical path length from the 50/50 beam splitter of $z_{S1}, z_{S2}, z_{S3} \dots z_{Sn}$. The field incident on the beam splitter after back-reflected/back-scattered from all depths of reflectors in the sample arm is given by $E_S = \frac{E_0}{\sqrt{2}} \sum_{n=1}^N r_{Sn} e^{i2kz_{Sn}}$.

The power from both the reference arm and the sample arm are halved upon passing

through the 50/50 beam splitter again and the interference signal detected at the detector is given by $I_D = \rho \langle \left| \frac{E_R}{\sqrt{2}} + \frac{E_S}{\sqrt{2}} \right|^2 \rangle = \frac{\rho}{2} \langle |(E_R + E_S)(\overline{E_R + E_S})| \rangle$. Here, ρ is the responsivity of the detector, the angular brackets in this equation denote the integration over the response time of the detector, and $\overline{(E_R + E_S)} = \overline{E_R} + \overline{E_S}$ which is one of the properties of complex conjugate.

When the surface of the beam splitter is set as $z = 0$ and the intensity at the detector could be expressed as

$$I_D(k, \omega) = \frac{\rho}{4} \left\langle \left| s(k, \omega) r_R e^{i(2kz_R - \omega t)} + s(k, \omega) \sum_{n=1}^N r_{Sn} e^{i(2kz_{Sn} - \omega t)} \right|^2 \right\rangle \quad (8)$$

Since the temporal frequency of light wave $\mathcal{V}$ oscillates much faster than detector's response time, it is reasonable to eliminate the terms dependent on ω ($\omega = 2\pi\mathcal{V}$). Thereafter, a real result for the detector current which is commonly known as “spectral interferogram” could be generated as follows:

$$\begin{aligned} I_D(k) = & \frac{\rho}{4} [S(k)(R_R + R_{S1} + R_{S2} + \dots + R_{Sn})] && \text{“DC Terms”} \\ & + \frac{\rho}{2} \left[S(k) \sum_{n=1}^N \sqrt{R_R R_{Sn}} \cos[2k(z_R - z_{Sn})] \right] && \text{“Cross-correlation Terms”} \\ & + \frac{\rho}{4} \left[S(k) \sum_{n \neq m=1}^N \sqrt{R_{Sn} R_{Sm}} \cos[2k(z_{Sn} - z_{Sm})] \right] && \text{“Auto-correlation Terms”} \end{aligned} \quad (9)$$

Where $S(k) = \langle |s(k, \omega)|^2 \rangle$ denotes the power spectrum depending on the light source, and $R_R = |r_R|^2$ and $R_{Sn} = |r_{Sn}|^2$ denote the power reflectivity of the reference reflector and sample reflector, respectively. In Eq. (9), three distinct components are included:

(1) The “DC” component which is a path length independent offset to the detector current. This term depends on the wave number spectrum of the light source with an amplitude which is proportional to the summation of the power reflectivity of the reflectors at reference arm and sample arm.

(2) The “cross-correlation” component for each sample reflector, which is a term desired for OCT imaging. It is scaled by both the wave number spectrum $S(k)$ of the light source

and the optical path length difference between the reference reflector and the sample reflectors $[(z_R - z_S)]$. The amplitude is determined by $\sqrt{R_R R_{Sn}}$, proportional to the square root of the reflectivity of the sample reflectors.

(3) An “auto-correlation” component indicates the interference that occurs between sample reflectors which appears as artifacts in typical OCT imaging system. This term depends on the reflectivity of the sample reflectors.

Except for these intuitive understandings of the intensity in OCT imaging, it is also useful to understand the effect of different source spectra and different sample reflectors on it. Firstly, we consider there is a single sample reflector, the intensity is given by $I_D(k) = \frac{\rho}{4} S(k) [(R_R + R_S) + 2\sqrt{R_R R_S} \cos[2k(z_R - z_S)]]$ and, thus, only the DC term and a single interferometric component are present. The light source spectrum $S(k)$ is modulated by one cosinusoid whose frequency is inversely related to the distance between the reference reflector and the sample reflector as illustrated in **Figure 2. 8**. Besides, the amplitude $\sqrt{R_R R_{Sn}}$ of the “modulated” spectrum (or the visibility of the spectral fringes) is determined by the reflectivity of the sample reflector $\sqrt{R_{Sn}}$ (**Figure 2. 8**).

In case of multiple reflectors which is the real-world condition in OCT imaging, the spectrum will be modulated by multiple cosinusoids. Each modulated spectrum has a period and an amplitude feature that is given by the corresponding sample reflector (**Figure 2. 8**). In addition, for multiple reflectors, autocorrelation component also appears, with frequency modulated by the optical delay between sample reflectors and amplitude determined by $\sqrt{R_{Sn} R_{Sm}}$. As the sample reflectivity is usually much smaller relative to the reference reflection, the autocorrelation component is usually smaller compared to the cross-correlation component. Also, as sample reflectors tend to be closely clumped together relative to the distance between the sample reflector and the reference reflector, the modulation frequency of autocorrelation term tends to be quite small.

Figure 2. 8 Key features of the spectral interferogram in OCT. For a single sample reflector (left), the intensity could be expressed as $I_D(k) = \frac{\rho}{4} S(k) [(R_R + R_S) + 2\sqrt{R_R R_S} \cos[2k(z_R - z_S)]]$. $(R_R + R_S)$ the amplitude of the DC term; the cross-correlation term with amplitude of $(2\sqrt{R_R R_S})$ and wave number period of $(\frac{\pi}{z_R - z_S})$ riding on top of the DC term (note that we leave out factors of $\frac{\rho}{4} S(k)$ for clarity). For multiple sample reflectors (right), the cross-correlation terms is a superposition of all cosinusoids. (This figure is reproduced from reference²¹).

In SD-OCT, all spectral components of the wave-number-dependent detector current ($I_D(k)$) are dispersed by a grating, captured by an array detector that is placed at the output of the spectrometer and finally processed using Fourier analysis. In this way, the internal sample reflectivity profile $\sqrt{R_S(z_S)}$ could be approximated and reconstructed, which is the aim of low-coherence interferometry. Note dispersing $I_D(k)$ by means of wave-number and its capturing depend on the experimental details of the grating and the detection apparatus, respectively.

Using the property of Fourier transform pair $[\frac{1}{2} [\delta(z + z_0) + \delta(z - z_0)] \xleftrightarrow{F} \cos kz_0]$ and the convolution property of Fourier transform $[x(z) \otimes y(z) \xleftrightarrow{F} X(k)Y(k)]$, the inverse Fourier transform of **Eq. (9)** in section 2.3.2 could be given as

$$\begin{aligned}
 i_D(z) = & \frac{\rho}{8} [\gamma(z)(R_R + R_{S1} + R_{S2} + \dots + R_{Sn})] && \text{“DC Terms”} \\
 & + \frac{\rho}{4} \left[\gamma(z) \otimes \sum_{n=1}^N \sqrt{R_R R_{Sn}} \delta[z \pm 2(z_R - z_{Sn})] \right] && \text{“Cross-correlation Terms”}
 \end{aligned} \tag{10}$$

$$+ \frac{\rho}{8} \left[\gamma(z) \otimes \sum_{n \neq m=1}^N \sqrt{R_{Sn} R_{Sm}} \delta[z \pm 2(z_{Sn} - z_{Sm})] \right] \quad \begin{array}{l} \text{“Auto-correlation} \\ \text{Terms”} \end{array}$$

Where, $\gamma(z)$ is achieved by Fourier transform of spectrum $S(k)$, which is called “coherence function” dominating the axial PSF for each reflector in OCT system. The Fourier transform between light source spectrum $S(k)$ and “coherence function” $\gamma(z)$ has been illustrated in the illustration of OCT axial resolution under **Section 2.2**. Clearly, the acquired sample field reflectivity profile given below is embedded within the cross-correlation term of **Eq. (10)**:

$$\sqrt{R_S(z_S)} = \sum_{n=1}^N \sqrt{R_{Sn}} \delta[z_S - z_{Sn}] \quad (11)$$

If we carry out the convolutions by making use of the sifting property of the delta function, interferometric measurement which is regarded as the “axial-scan” or “A-scan” could be obtained:

$$\begin{aligned} i_D(z) = & \frac{\rho}{8} [\gamma(z)(R_R + R_{S1} + R_{S2} + \dots + R_{Sn})] \\ & + \frac{\rho}{4} \sum_{n=1}^N \sqrt{R_R R_{Sn}} [\gamma(2(Z_R - Z_{Sn})) + \gamma(-2(Z_R - Z_{Sn}))] \\ & + \frac{\rho}{8} \sum_{n \neq m=1}^N \sqrt{R_{Sn} R_{Sm}} [\gamma(2(z_R - z_{Sn})) + \gamma(-2(z_R - z_{Sn}))] \end{aligned} \quad (12)$$

The results for discrete sample reflectors and “A-scan” with a Gaussian-shaped light spectrum could be illustrated in **Figure 2. 9**. As plotted in the figure, the sample field reflectivity profile $\sqrt{R_S(z_S)}$ could be restored in the cross-correlation terms with the following modifications:

- (1) The sample reflectivity profile $\sqrt{R_S(z_S)}$ is a function of the reference coordinate z_R instead of the sample coordinate z_S ; that is, the zero position corresponds to the distance equal to reference arm reflector;
- (2) The apparent displacement of each sample reflector from the reference reflector is doubled which means that a round-trip distance to each reflector is measured by the interferometer, so a new single-pass depth variable is defined ($\hat{z} = 2z$) to rescale axial

depth to account for the factor of 2;

(3) Each sample reflector appears blurred out to a width of a coherence length by convoluting with the function of $\gamma(z)$. Thus, using source with bandwidth as broad as possible (low coherence length), the fidelity of the sample reflector profile will increase;

(4) The detected sample reflectivity is usually very small but is amplified by the homodyne gain factor which is represented by strong reference reflectivity.

In conclusion, these modifications can be dealt with by interpreting the data properly. In addition, artifacts such as mirror-image artifact arising from the cross-correlation terms and artifacts arising from the DC terms and the autocorrelation terms also result in modification to the field reflectivity profile and are generally more serious. Approaches have been developed to eliminate these artifacts.

Figure 2. 9 Illustration of discrete-reflector sample field reflectivity function (top) and an “A-scan” (bottom) in SD-OCT. (This figure is reproduced from reference²¹).

2.4 Sensitivity Fall-off in Spectral-domain OCT

The above-mentioned spectral interferogram $I_D(k)$ of FD-OCT and its corresponding Fourier transform $i_D(z)$ in **Section 2.3** illustrate the fundamental principle underlying

SD-OCT imaging system. Whereas, in practical conditions, the spectral interferogram is captured by devices having real-world limitations and is typically recorded by a “sampling” operation so that the digital signal computation of its Fourier transform could be conducted rapidly. The conceptual effects of spectral resolution and sampling operation upon the spectral interferogram and A-scan are illustrated in **Figure 2. 10**.

Figure 2. 10 Sensitivity “falloff” and maximum imaging depth in SD-OCT.

The depth-dependent sensitivity falloff $\hat{z}_{6dB}$ is correlated with the spectral resolution of the interference spectrum, which is determined by the spectrometer resolution $\delta_r k$ of SD-OCT. (This figure is reproduced from reference²¹).

First of all, the spectral interferogram is always acquired by an instrumentation having limited spectral resolution $\delta_r k$ (or $\delta_r \lambda$) which in SD-OCT is the spectral resolution of the spectrometer such as the finite spacing of the CCD pixels. As a result, the spectral interferogram may be degraded owing to the finite spectral resolution. The effect of the finite spectral resolution can be modeled by convolving the ideal spectral interferogram $I_D(k)$ from Eq. (9) with a Gaussian function whose FWHM is $\delta_r k$. Here, the Gaussian function could therefore be expressed as $g(k) = \frac{2\sqrt{\ln 2}}{\Delta k \cdot \sqrt{\pi}} e^{-\frac{4 \ln 2 \cdot k^2}{\delta_r k^2}}$. Given the convolution property $\mathbf{F}[f(x) \otimes g(x)] = \mathbf{F}[f(x)] \cdot \mathbf{F}[g(x)]$, the processed A-scan data is then expressed as the ideal A-scan $i_D(z)$ multiplied by a sensitivity fall-off (or sensitivity “rolloff”) factor, whose shape could be achieved by Fourier transforming operation of the Gaussian-shaped spectral resolution factor:

$$i_D(\hat{z}) \cdot \exp\left[-\frac{\hat{z}^2 \cdot \delta_r k^2}{4 \ln 2}\right] \xleftrightarrow{\mathbf{F}} I_D(k) \otimes \frac{2\sqrt{\ln 2}}{\Delta k \cdot \sqrt{\pi}} \exp\left[-\frac{4 \ln 2 \cdot k^2}{\delta_r k^2}\right] \quad (13)$$

Here, we use the rescaled depth variable $\hat{z} = 2z$ to remove the apparent depth-doubling factor and to allow the processed A-scan data to compare with the sample structure directly. As demonstrated in the exponential terms, the “roll-off” of sensitivity with depth could be understood as the decreasing visibility of higher fringe frequencies corresponding to large sample depths. The sensitivity falloff is quantified as the one-sided depth range at which the sensitivity decreases by a factor of 1/2 (so that $\exp\left[-\frac{\hat{z}^2 \cdot \delta_r k^2}{4 \ln 2}\right] = \frac{1}{2}$), which is 6-dB in SNR units:

$$\hat{z}_{6dB} = \frac{2 \ln 2}{\delta_r k} = \frac{\ln 2}{\pi} \frac{\lambda^2}{\delta_r \lambda} \quad (14)$$

Here, $\hat{z}_{6dB}$ is given in both wave number ($\delta_r k$) and wavelength ($\delta_r \lambda$) terms and the latter is recognizable as 1/2 of the coherence length corresponding to the spectral resolution.

Secondly, in real-world processing of spectral interference data in SD-OCT, the computer-based device usually “sampling” the spectral interferogram for rapid digital computation of its Fourier transform. The spectral interferogram is sampled with sampling interval $\delta_s k$ into M channels linearly spaced in the k -domain. Thus, the total

collected wave number range is $\Delta k = M \cdot \delta_s k$ and the sampling interval in the z -domain is then set to be $\delta_s \hat{z} = 2\pi/(2\Delta k) = \pi/(M \cdot \delta_s k)$ where the factor of 2 in the denominator is due to the use of the rescaled depth parameter $\hat{z}$. Given by the Nyquist criterion, the maximum sampling depth are

$$\pm \hat{z}_{max} = \pm \frac{\pi}{2 \cdot \delta_s k} = \pm \frac{\lambda^2}{4 \cdot \delta_s \lambda} \quad (15)$$

A summary of these parameters is provided in **Table 2. 1**. In addition to these two limitations which are difficult to overcome, some additional real-world complications can arise and most of them could be handled with appropriate signal processing. For example, dispersion effects owing to mismatched optical path lengths between sample arm and reference arm may be corrected by adding appropriate phase factors to the interferometric data prior to the Fourier transforming operation.

Table 2. 1 Effects of finite spectral resolution and sampling in SD-OCT

	6-dB sensitivity roll-off point	Maximum one-sided imaging depth
	$\hat{z}_{6dB}$	$\hat{z}_{max}$
Wavenumber units	$\frac{2 \ln 2}{\delta_r k}$	$\frac{\pi}{2 \cdot \delta_s k}$
Wavelength units	$\frac{\ln 2}{\pi} \frac{\lambda^2}{\delta_r \lambda}$	$\frac{\lambda^2}{4 \cdot \delta_s \lambda}$

Note: $\delta_r k$ and $\delta_r \lambda$: the spectral resolution of the spectrometer; $\delta_s k$ and $\delta_s \lambda$: the spectral sampling interval in signal processing; depth listed here are in free space and should be divided by the refractive index in media.

2.5 Signal-to-Noise Ratio in Spectral-Domain OCT

As mentioned at the beginning of **section 2.3**, SD-OCT has a dramatic improvement in imaging speed as well as sensitivity which is often used interchangeably with signal-to-noise (SNR) if light attenuation is not taken into consideration (i.e. sample surface is assumed as a perfect reflector) comparing to TD-OCT. SD-OCT has increased

sensitivity or SNR significantly based on the fact that the noises in particular the shot noise are dramatically reduced by replacing the single-element detector with a multi-element array detector. Specifically, in a single-detector TD-OCT imaging system, the shot noise produced by the power at a particular wavelength is present at all frequencies and thus affects the SNR at all other wavelengths. Whereas, in SD-OCT that uses multi-element array detector, the cross effect of shot noise is eliminated by spectrally dispersing each wavelength to a separate detector. The significantly improved sensitivity or SNR (~2-3 orders of magnitude) of SD-OCT over TD-OCT allows for faster image acquisition speeds but without compromising image quality^{22,23}.

In this section, to theoretically demonstrate the sensitivity of a SD-OCT system, I illustrate SNR of SD-OCT which is usually expressed in decibel units (dB) and denotes the minimum detectable reflected optical power relative to a perfect reflector. SNR is defined as the ratio of squared signal power ($\langle I_D \rangle^2$) to noise process variance (σ^2) ($SNR = \frac{\langle I_D \rangle^2}{\sigma^2}$). Complete SNR analysis includes the evaluation of optical power incident on the detector and consideration of different possible noise sources. The signal and noise terms here are expressed in terms of charge squared [e^2] to facilitate the analysis of noise in cases of detectors using CCDs. Only the real-part of the complex cross-correlation term of the spectral density is achieved, contributing to a detected signal given by

$$S_{SD} = \frac{\eta^2 e^2 P_{ref} P_{sample} \tau_i^2}{E_\nu^2} [e^2] \quad (16)$$

Here, η denotes the detector efficiency; e is the electron charge; P_{ref} and P_{sample} is the reference arm power and sample arm power per detector element respectively; τ_i represents the integration time and E_ν the photon energy.

Noises including the readout noise, dark noise, shot noise (σ_{shot}^2), and relative intensity noise (σ_{RIN}^2) contribute to the overall noise of the detector per readout cycle and per detector element:

$$\begin{aligned} \sigma_{noise}^2 &= \sigma_{r+d}^2 + \sigma_{shot}^2 + \sigma_{RIN}^2 \\ &= \sigma_{r+d}^2 + \frac{\eta e^2 P_{ref} \tau_i}{E_\nu} + \left(\frac{\eta e P_{ref}}{E_\nu} \right)^2 \tau_i \tau_{coh} [e^2] \end{aligned} \quad (17)$$

Here, the reference arm power is assumed to be much higher than that of the sample arm; σ_{r+d}^2 denote the sum of readout noise and dark noise; τ_i represents the detector integration time, $\tau_{coh} = \sqrt{\frac{2 \ln 2}{\pi}} \frac{\lambda_c^2}{c \cdot \delta \lambda}$ denotes the coherence time with c the light speed and λ_c the center wavelength of the light source. When the shot noise dominants both readout-dark noises and relative intensity noise (i.e. $\sigma_{shot}^2 / \sigma_{r+d}^2 > 1$ and $\sigma_{shot}^2 / \sigma_{RIN}^2 > 1$), the optimal SNR performance will be achieved. When the readout-dark noises are equal to the relative intensity noise ($\sigma_{r+d}^2 = \sigma_{RIN}^2$), the optimal reference-arm power is found ($P_{ref} = \frac{\sigma_{r+d} E \nu}{\eta e \sqrt{\tau_i \tau_{coh}}}$).

For a SD-OCT system to operate close to the shot-noise-limit performance, shot noise should dominate over readout-dark noise and relative intensity noise (RIN) respectively at the optimal reference arm power. Under this circumstance, the inequalities described above could also be expressed as

$$\frac{e \sqrt{\tau_i}}{\sigma_{r+d} \sqrt{\tau_{coh}}} > 1 \quad (18)$$

In general, we expect the coherence time τ_{coh} of the detector to be as long as possible and the integration time τ_i as short as possible. Specifically, the coherence time τ_{coh} is inversely proportional to the spectral resolution of the spectrometer ($\tau_{coh} = \sqrt{\frac{2 \ln 2}{\pi}} \frac{\lambda_c^2}{c \cdot \delta \lambda}$), which in turn linearly relates to the maximum ranging depth of the system because the maximum ranging depth is also inversely proportional to the spectral resolution of the spectrometer ($\pm z_{max} = \pm \frac{\lambda_c^2}{4 \cdot \delta \lambda}$). Thus, a longer coherence time allows higher maximum ranging depth. Decreasing the integration time permit an increase in the reference arm power without saturating the detector which will consequently reduce the autocorrelation noise induced by interference within the sample in an ultrahigh-speed SD-OCT. Conclusively, the system performance is mostly determined by the readout and dark noise (σ_{r+d}) of the detector as presented in **Eq. (18)**.

Thus, the SNR of SD-OCT system that operates in the shot noise limit performance is shown to be

$$SNR_{SD} = \frac{S_{SD}}{\sigma_{noise}^2} = \frac{\frac{\eta^2 e^2 P_{ref} P_{sample} \tau_i^2}{E_{\nu}^2}}{\frac{\eta e^2 P_{ref} \tau_i}{E_{\nu}}} = \frac{\eta P_{sample} \tau_i}{E_{\nu}} \quad (19)$$

As shown in **Eq. (19)**, in the shot-noise-limit, SNR of SD-OCT seems to be independent of the spectral bandwidth of the light source, which implies that the axial resolution can be improved at no penalty to the system's SNR, provided that the full spectral bandwidth is imaged onto an array detector, i.e. CCD. However, it is worth mentioning that the sample arm power in **Eq. (19)** is assumed to come from a single sample reflecting surface. Whereas, in biological tissue, the back-reflected power should come from multilayered structures along the depth profile and the SNR of a position along the depth is given on average by the total power reflected by all depths within the coherence length of the light source. Thus, as the axial resolution increases, the coherence length decreases which result in decrease of the total back-reflected power within the light coherence length. Consequently, the SNR at a particular position will reduce as the axial resolution increase.

2.6 Transverse Image Formation in Spectral-Domain OCT

2.6.1 Sample arm in SD-OCT system

Figure 2. 11 shows a schematic of a fiber-based SD-OCT imaging system. Polychromatic light is directed into a fiber coupler by a single mode fiber. Here, the fiber coupler serves as the beam splitter which divides the incident light into reference arm and sample arm, respectively. Light existing the reference fiber is incident upon the reference mirror by passing through a collimation lens and an objective lens. Similarly, light that exists the sample fiber is directed to the sample by a collimation lens, an objective lens and a galvo scanner which is used for transverse scanning. Thereafter, light reflected from the reference mirror and backreflected/backscattered from the sample is redirected back into the fiber coupler and interfere with each other. The spectral interference pattern is then directed to the spectrometer where it is dispersed by a diffraction grating as a function of wavelength and recorded by a linear camera.

Rescaling the spectrometer output from wavelength to frequency and performing Fourier transform, an axial-scan at the position of the focal spot is established.

Figure 2. 11 A schematic of fiber-based SD-OCT imaging system. L: lens, SMF: single mode fiber, GV: galvo scanner, CCD: charge-coupled detector.

2.6.2 Lens: Fourier transforming property

As plotted in **Figure 2. 11**, in the sample arm, the sample is placed near the back focal plane of the objective lens and the lenses play an important role in directing light propagation. To perform numerical analysis of the light intensity back-scattered from the sample, I will first illustrate the Fourier transforming property of a lens.

Figure 2. 12 Diagram of lens geometry.

As illustrated in **Figure 2. 12**, assuming the objective lens is thin, spherical lens, we establish coordinate systems at the pupil plane and the focal plane respectively. With

the paraxial approximation, we know that the phase delay introduced by the lens is given by

$$\phi(x, y) = -k \frac{x^2 + y^2}{2f} \quad (20)$$

where f is the focal length of the lens. Assuming the electric field of the parallel light wave that just before the objective lens is $E(x_1, y_1)$ which should be a constant if the parallel light incidents on the lens perpendicularly, the field just after the lens $E'(x_1, y_1)$ is given by

$$E'(x_1, y_1) = E(x_1, y_1)P(x_1, y_1)e^{i\phi(x_1, y_1)} \quad (21)$$

where $P(x_1, y_1)$ is the pupil function of the lens which should be a constant for a perfect lens. With Fraunhofer approximation, the field of the light at the focal plane is

$$\begin{aligned} E(x_2, y_2) &= \frac{1}{i\lambda f} \iint_{-\infty}^{+\infty} E'(x_1, y_1) e^{ik\sqrt{(x_2-x_1)^2+(y_2-y_1)^2+\Delta z^2}} dx_1 dy_1 \\ &\approx \frac{1}{i\lambda f} \iint_{-\infty}^{+\infty} E'(x_1, y_1) e^{ik\left(f+\frac{(x_2-x_1)^2+(y_2-y_1)^2}{2f}\right)} dx_1 dy_1 \\ &= \frac{e^{ik\left(f+\frac{x_2^2+y_2^2}{2f}\right)}}{i\lambda f} \iint_{-\infty}^{+\infty} E(x_1, y_1)P(x_1, y_1)e^{-i2\pi\left(\frac{x_1x_2+y_1y_2}{\lambda f}\right)} dx_1 dy_1 \end{aligned} \quad (22)$$

Therefore, the **Eq. (22)** could also be expressed in terms of a Fourier Transform:

$$E(x_2, y_2) = \frac{e^{ik\left(f+\frac{x_2^2+y_2^2}{2f}\right)}}{i\lambda f} \mathbf{F}[E(x_1, y_1)P(x_1, y_1)]|_{v_x=\frac{x_2}{\lambda f}, v_y=\frac{y_2}{\lambda f}} \quad (23)$$

Where $\mathbf{F}$ represents Fourier transform operation. Clearly shown in **Eq. (23)**, the electrical field at the focal plane of the lens is the Fourier transform of that incident on the lens which illustrates the Fourier transforming property of a lens.

2.6.3 Image formation at the sample arm

Here, considering the light incident on the sample with a field point spread function of

$h(x, y)$ and the field reflectivity of the sample being $\sqrt{R_s}(x, y)$, the image intensity that reflected from the sample is given by

$$I_s = |h(x, y) \otimes \sqrt{R_s}(x, y)|^2 \quad (24)$$

Where $\otimes$ means the convolution operation. In this thesis, numerical analysis is used to simulate the reflectance from the subcellular structures within the tissues illuminated by the OCT imaging system.

Figure 2. 13 Schematic of the sample arm of an OCT system. SMF: single mode fiber, GV: galvo scanner.

In the sample arm of the OCT system, light back-reflected/back-scattered from the sample is redirected to the single mode fiber after passing through two lenses (**Figure 2. 13**). Thus, the image intensity collected by the sample fiber is

$$I = |\mathbf{F}\{\mathbf{F}[h(x, y) \otimes \sqrt{R_s}(x, y)]\}|^2 \quad (25)$$

Where $\mathbf{F}$ represents Fourier transform operation. Therefore, as presented in **Eq. (25)**, the square root of power reflectivity ($\sqrt{R_s}$) could be derived from the intensity measured by the detector.

Summarily, in this chapter, an introduction of the working principle of SD-OCT and a detailed theory on axial and transverse resolution, and image formation (confocal optics), and sensitivity fall-off were provided. The theories on spatial resolutions guide us in the optical system design, fabrication and testing in Chapter 3. The theories on sensitivity fall-off lay the foundation of interferometer-in-spectrometer in Chapter 3. The theories on imaging formation are the foundation of simulating the backscattered signal from various nanometer scale subcellular components in Chapter 5.

Chapter 3

OCT System Development and Construction

3.1 Micro-OCT system

3.1.1 Introduction

Micro-OCT (μ OCT) is a type of SD-OCT system whose spatial resolution has been pushed to the cellular level (1-2 μ m), making it possible to resolve histopathological details within biological tissues in real-time without the need of invasive biopsy and time-consuming tissue preparation and processing¹⁹. μ OCT has all those advantages of SD-OCT which has replaced its predecessor TD-OCT owing to its superior sensitivity combined with the lack of need for a mechanical scanning in the reference arm, opening up the possibilities of faster scanning without loss of image qualities.

Whereas, the μ OCT system reported in previous literatures is mainly composed of free-space optics which is prone to misalignment in the optical paths due to vibration during transportation or operation. In addition, the benchtop probe in the sample arm consists of bulky optical elements which makes it cumbersome to handle for imaging *in vivo* or in clinical suites. To deal with these problems, our group has developed an improved version, fiber-based μ OCT system and I focus on the design and development of a compact handheld probe, making the system suitable for *in vivo* use.

3.1.2 Materials and Methods

Figure 3. 1 illustrates the construction of a fiber-based μ OCT system composed of a compact fiber-based imaging console and a flexible handheld probe. In brief, a

supercontinuum light source (SC-5, Yangtze Soton Laser, Wuhan, China) provided a broadband illumination from 650 nm to 980 nm centered at ~800 nm. The output of the light source was coupled into a single-mode fiber (630-HP, Thorlabs, USA) and split into a sample fiber (630-HP, Thorlabs, USA) and a reference fiber (630-HP, Thorlabs, USA) through a 50:50 free-space beamsplitter (BS008 and PAFA-X-4-B, Thorlabs, USA). The light back reflected from the reference arm and backscattered from the sample arm were recombined by the same beamsplitter and directed into a high-speed spectrometer through a single-mode fiber (630-HP, Thorlabs, USA). The spectrometer was composed of an achromat collimator ($f = 30$ mm, AC127-030-B, Thorlabs, USA), a transmission grating (960 l/mm, WP-960/840, Wasatch Photonics, USA), a camera lens (AF Nikkor 85 mm f/1.8D, Nikon, Japan), and a line scan camera (EV71YEM4CL2014-BA9, E2V, USA) with a pixel size of $14\ \mu\text{m} \times 28\ \mu\text{m}$ (width $\times$ height) and a pixel number of 2048. The output of the camera was transferred to the computer via an image acquisition board (KBN-PCE-CL2-F, Bitflow, USA).

Figure 3. 1 A Schematic of μ OCT imaging system. L1-L8: optical lenses; BS: beamsplitter; SMF: single mode fibre; GS: galvanometer scanning mirrors; PC:

polarization controller; RM: reference mirror; CL: camera lens; LSC: line scanner camera; IMAQ: image acquisition board; AO: analog outputs.

The sample arm as illustrated in **Figure 3. 1** was constructed as a flexible probe to conduct OCT imaging of biological tissues *in vivo* and *ex vivo*. The sample light from the single mode fiber (630-HP, Thorlabs, USA) was collimated by an achromat ($f = 15$ mm; AC050-015-B-ML, Thorlabs, USA) and focused by a long-working-distance near-infrared objective lens ($f = 10$ mm; M Plan Apo NIR 20X, Mitutoyo, Japan).

Figure 3. 2 Geometry of window holder. The three dimensions of the window holder (top dashed box) and photographs of the window holder before (bottom left) and after (bottom right) mounted onto a half-inch (diameter = 12.7 mm) tube. FS: Fused Silica.

We positioned a glass window (fused silica with anti-reflection coating, 1 mm thick) $\sim 100\ \mu\text{m}$ before the focal plane to maintain the working distance between the objective lens and the epithelium. The glass window is 2 mm in diameter and mounted on a three-dimensional (3D) printed tapered holder (**Figure 3. 2**) for accessing to the tissue surface *in vivo*. For *ex vivo* studies, this flexible sample arm probe was mounted onto a microscope frame. The light power on the tissue was 5.5 mW and 1.8 mW for *ex vivo* and *in vivo* studies, respectively.

Figure 3. 3 presents the fiber-based μOCT imaging system setup. Each μOCT cross-sectional image contained maximally 2048 axial lines and the data size of each 3D image was $1024 \times 1024 \times 2048$ ($x \times y \times z$). The corresponding tissue volume was $1.744\ \text{mm} \times 1.744\ \text{mm} \times 0.88\ \text{mm}$ (width $\times$ width $\times$ depth in water (refractive index = 1.33)). We used an axial line rate of 20k Hz (20 frames/sec.) and 60k Hz (60 frames/sec.) for *ex vivo* and *in vivo* studies, respectively.

Figure 3. 3 A photograph of fiber-based μOCT system. Left: the imaging console of the μOCT system; right: a flexible probe denoting the sample arm of the system.

3.1.3 Experimental Results

The spatial resolutions and the 6-dB axial range were evaluated. The sample fiber emitted a Gaussian light beam centered at ~ 800 nm. The nominal numerical aperture of the fiber is 0.13 (1% fit) and the corresponding mode field diameter is $4.0 \pm 0.5 \mu\text{m}@630$ nm ($1/e^2$ fit). The light propagated through a collimation lens ($f = 15 \mu\text{m}$) and an objective lens ($f = 10 \mu\text{m}$), enabling a calculated FWHM lateral resolution of $1.77 \mu\text{m}$ and a depth of focus of $17.7 \mu\text{m}$ in air. The lateral resolution was also assessed using a USAF resolution chart (R1DS1P - Positive 1951 USAF Test Target, Thorlabs) and the resolution chart bar of $2.2 \mu\text{m}$ (228.1 black-white line pairs / millimeter) could be clearly resolved by the μOCT system (**Figure 3. 4a**).

Figure 3. 4 Spatial resolutions and ranging depth of μOCT system. (a) An *en face* image of USAF resolution chart. The 1-6 elements in group 7 was presented in the image and resolution chart bar black-white pairs of $4.4 \mu\text{m}$ can be clearly resolved. (b) Axial point spread functions measured over the optical path delay of 0-800 μm in air. Zoomed-in view shows the axial point spread function at the depth of approximately 300 μm and axial resolution is measured to be 1.8 μm in air. Ranging depth here is defined as the 6-dB fall-off corresponding to the

optical path delay where the intensity falls off to 1/4 of its maximum at the zero optical path delay.

To experimentally evaluate the axial ranging depth, we acquired interferograms at different optical path delay. Specifically, we first positioned a partial reflector at the focus of the objective lens in the sample arm; we then acquired an interferogram every 50 μm by changing the optical path length of the reference arm away from that of the sample arm. The axial resolution measured from the FWHM of the axial point spread function, as illustrated in **Figure 3. 4b**, was $\sim 1.8 \mu\text{m}$ in air. The ranging depth, quantified as the 6-dB roll-off or when the peak of the interference intensity falls to one-half of its maximum, was measured to be $\sim 800 \mu\text{m}$ in air (**Figure 3. 4b**).

We also evaluated the axial field of view by recording sample arm intensity when the sample reflector was moved away from its focus with an interval of 5 μm . The axial field of view, defined as the double-sided axial range of 6-dB intensity roll-off, was measured to be $\sim 110 \mu\text{m}$ (**Figure 3. 5**).

Figure 3. 5 Plot of axial field of view of μOCT system. The peak values of the sample arm intensity were recorded when the sample reflector placed at the focus of the objective lens was moved away from its focus. The axial field of view were the two-sided axial range when sample arm intensity falls to 1/4.

3.2 Interferometer-in-spectrometer OCT system

3.2.1 Background and Motivation

In the time-domain OCT, maintaining a high spectral bandwidth of the detected signal is relatively straightforward since the detectors are photodiodes. In this case, typically the spectral response of available photodiodes is broad enough to support ultrahigh resolution OCT, and there is no aberration issues and other band-limiting components²⁵. However, it is challenging to maintain a high detected spectral bandwidth in the FD-OCT due to the spectral response limitations of the optics components in the detection arm.

Generally, FD-OCT includes two types: SS-OCT and SD-OCT. The former is becoming popular owing to its superior instantaneous spectral line width and line rate over the latter. However, it is very difficult, if possible, to achieve an ultrahigh axial resolution (1-2 μm) using a swept source, because of the limitation of the gain medium in the spectral bandwidth (up to 140 nm)²⁶. As a result, currently SD-OCT is the only option for ultrahigh or cellular resolution imaging with the accumulating achievements of broadband light sources^{19,27-29}. Whereas, although broadband light sources are available, SD-OCT detects spectral interference signals by use of a spectrometer which is the most significant bandwidth-limiting device in the system and may thus compromise the axial resolution ($\Delta z = \frac{2 \ln 2}{\pi} \frac{\lambda_c^2}{\Delta \lambda}$) and the ranging depth ($\hat{z}_{6dB} = \frac{\ln 2}{\pi} \frac{\lambda^2}{\delta_r \lambda}$). Specifically, currently available gratings and line scan cameras used for OCT spectrometers are limited in the spectral bandwidth³⁰ which suffer from trade-offs between the detected spectral bandwidth and the spectrometer efficiency. For example, the Si-based camera has a poor response above ~900 nm and InGaAs-based camera is particularly suboptimal in the spectral region under 950 nm. As a result, in order to maintain a detected spectral bandwidth high enough to support an ultrahigh axial resolution (1-2 μm), the spectrometer efficiency or detection sensitivity has to be sacrificed, which is not viable for clinical applications.

A straightforward solution is to use a spectral shaping measure to compensate for the bandwidth limitation. Apparently, it is not reasonable to shape the detected light in the sample arm since the scattered power budget is extremely tight as the optical power scattered from the sample arm is proportional to sensitivity. Shaping the input light is not desired since it is not power economical. The only light that can be sacrificed is that in the reference beam, simply because in a typical OCT system the majority of light allocated to the reference arm is wasted or attenuated (**Figure 3. 6A**). Therefore, one

can insert a spectral shaper in the reference arm to improve the axial resolution without compromising the detection sensitivity. However, a more cost-effective solution is to use the grating in the spectrometer as the spectral shaper.

In this study, we have developed a novel SD-OCT design which integrated the interferometer into the spectrometer, termed interferometer-in-spectrometer (IIS) scheme (**Figure 3. 6B**). IIS scheme uses the grating in the spectrometer as the spectral shaper and the terminal facet of the core of the spectrometer input fiber as the interferometer, so that the reference spectrum can be shaped to compensate the limited spectral bandwidth of the sample light through interference. This IIS scheme provides a platform that can support other optical manipulations for improving performance of SD-OCT.

3.2.2 Materials and Methods

In this part of work, we used an ultrahigh resolution OCT system with conventional interferometer as the benchmark. Shown in **Figure 3. 6a**, a broadband light source (Superlum Broadlighters M-T-850-HP, Ireland) with a center wavelength of 850 nm and a FWHM bandwidth of 165 nm was used. The source output was split by a broadband 50:50 fiber coupler (TW850R5A2, Thorlabs Inc., USA) with single-mode fiber SMF-780HP which served as the interferometer of the system and directed light to the sample arm and the reference arm respectively. The sample arm consisted of a collimation lens L1 (LA4647-B-f = 20, Thorlabs Inc., USA), a galvo scanner (GVSM002/M, Thorlabs Inc., USA) and an objective lens L2 (AC127-030-B, Thorlabs Inc., USA). The reference arm was equipped with the same refractive optics as the sample arm. Light back-reflected/backscattered from the sample and reference arm was then recombined by the same 50:50 fiber coupler, so that light from both arms interfered with each other. Thereafter, the interference signal was directed to the spectrometer through the spectrometer input fiber of the coupler. The spectrometer (**Figure 3. 6a**, blue dashed box) was composed of a collimation lens L5 (AC127-030-B, Thorlabs Inc., USA), a grating (1200 l/mm, WP-1200/840-50.8, Wasatch Photonics Inc., USA), a camera lens (Nikon AF Nikkor 85 mm f/1.8D), and a line scan camera (CCD, E2V, EV71YEM4CL2014-BA9) with a pixel size of $14\ \mu\text{m} \times 28\ \mu\text{m}$ (width $\times$ height) and a pixel number of 2048. In this standard OCT system, light went through the collimation

lens L5, the first-order diffraction channel of the grating and captured by the CCD of the camera. In this standard system, optical components including fiber coupler, grating and camera all behaves like band-pass filters, limiting the spectral bandwidth of detected light which thereafter degrade axial resolution and ranging depth.

Figure 3. 6 Schematics of ultrahigh resolution OCT imaging system. (a). Standard scheme; (b). IIS scheme. PC: polarization controller; L: lens; CL: camera lens; CCD: charge coupled device (line scan camera); DC: dispersion compensator (fused silica glass); IMAQ: image acquisition module.

In the IIS scheme, interference happens at the terminal facet of the spectrometer input fiber that's why it was called interferometer-in-spectrometer. Briefly, the reference light was collimated by lens L4, went through the zero-order channel of the grating, and focused to the terminal facet of the spectrometer input fiber by lens L5 (**Figure 3. 6b**, blue dashed box). Since L4 and L5 were identical, the focal spots of the reference beam and the back-reflected or back-scattered sample beam could overlap perfectly at the terminal facet of the core of the spectrometer input fiber (**Figure 3. 6b**, red arrow), so that the interferometer was integrated in the spectrometer. In this way, the spectrum degradation at the lower and longer wavelength of the reference arm owing to the

bandpass effect of the grating could be mitigated. The fiber terminal was flat-polished, so that the reference light reflected by the fiber terminal facet and the sample light transmitted through the fiber terminal facet propagated along the same direction. The interference light was collimated by L5, and directed to the line scan camera through the first-order channel of the grating and the camera lens, the same as that in the standard scheme. Since the interference signal intensity is the product of the power spectral density of the two arms according to $I_D(k) \propto \rho S(k) \sqrt{R_R R_S} \cos[2k(Z_R - Z_S)]$ (here, ρ donates the spectrometer response, $S(k) = \sqrt{S_S(k) S_R(k)}$), interference spectral bandwidth could be restored. The optical power from the reference fiber was measured to be 8 mW, which was attenuated to $\sim 400 \mu\text{W}$ by the grating and further to $\sim 16 \mu\text{W}$ by the fiber terminal facet reflection with the sample arm being blocked. A reference power of $\sim 16 \mu\text{W}$ was more than enough to support a line scan rate of up to 50 kHz.

In our experimental setup, both schemes shared the same sample arm and the spectrometer so that the measured results can be directly compared without bias.

3.2.3 Experimental Results

The spectrum of the light source was in an approximately top-hat shape with uniform power density of all the peaks (**Figure 3. 7a**, blue dashed curve). However, in a standard OCT system, the spectral response of the camera decreased approximately linearly from the short wavelength ($\sim 750 \text{ nm}$) to the long wavelength ($\sim 950 \text{ nm}$) by $\sim 75\%$ (**Figure 3. 7a**, cyan dash-dotted curve). To make things worse, the diffraction efficiency of the grating (i.e. the efficiency of the first-order diffraction channel of the grating) is essentially a spectral band-pass curve with 1-dB cut-off approximately at 740 nm and 930 nm, i.e. the first-order transmission efficiency is lower in the shorter and longer ends of the spectrum than in the center (data not shown). These spectral modulations resulted in a deteriorated detected sample spectrum with 40% smaller bandwidth and blue-shifted center wavelength (**Figure 3. 7a**, red dotted curve vs blue dashed curve).

In the IIS scheme, prior to interfering with the sample light, the reference light passed through the zero-order channel of the grating, which had much higher transmission efficiency in the short and long ends of the wavelength spectrum than that in the center. Therefore, the spectrum degradation owing to the bandpass effect of the grating could

be mitigated. As a result, the detected reference spectrum was restored at the lower and longer wavelength and presented a spectral shape of a notch filter (**Figure 3. 7a**, green solid curve), which accordingly restored the interference spectrum bandwidth.

Figure 3. 7 Detected spectra and axial point-spread functions. (a) The detected spectra from the standard scheme (red dotted curve) and the IIS scheme (green solid curve), respectively with the sample arm being blocked. The arrows indicate the locations of the spectrum that were affected by both the camera response and the grating transmission efficiency in the standard scheme. By contrast, the shorter and longer wavelength intensity were significantly increased after spectral shaping in the IIS scheme. The blue dashed curve represents the source spectrum and the cyan dash-dotted curve depicts the spectral response of the line scan camera. (b) The measured axial point-spread functions of the standard scheme (red dotted curve) and the IIS scheme (green solid curve), respectively. The depth scales are in air.

In the standard scheme, the axial resolution was measured to be 2.55 μm in air (**Figure**

3. 7b, red dotted curve) and the 6-dB intensity roll-off was 650 nm (**Figure 3. 8a**). In the IIS scheme, the FWHM axial resolution of the IIS scheme was measured to be 2.37 μm in air (**Figure 3. 7b**, green solid curve), which is $\sim 10\%$ higher than that of the standard scheme. The measured 6-dB intensity roll-off was at 800 μm in tissue (refractive index = 1.38) for the IIS scheme (**Figure 3. 8b**), which was 150 μm ($\sim 25\%$) longer than that in the standard scheme. Note that in the standard scheme the axial resolution (**Figure 3. 7b**) did not drop as much as the detected spectral bandwidth because of the blue shift of the center wavelength (**Figure 3. 7a**). However, the price to pay is the shorter ranging depth (**Figure 3. 8**) due to the large attenuation in the long wavelength region (**Figure 3. 7**).

Figure 3. 8 Axial point-spread functions over depth detected by changing the optical pathlength difference between the sample and the reference arms. (a). Axial point-spread functions of the standard scheme showing a 6-dB roll-off range of 650 μm in tissue (refractive index = 1.38). (b). Axial point-spread functions of the IIS scheme showing a 6-dB roll-off range of 800 μm in

tissue (refractive index = 1.38).

In order to demonstrate the imaging capability of IIS scheme, we acquired images of swine cornea using both the standard and the IIS scheme *ex vivo*. The swine eyes were harvested freshly from a local slaughterhouse, and was maintained in neutral buffered phosphate buffered saline during the transportation. The imaging experiments were conducted after 3-4 hours postmortem. We used an A-line rate of 20k Hz and a frame rate of 20 Hz. The transverse scanning range was 2.62 mm.

In the image acquired using the standard scheme (**Figure 3. 9a**), some corneal layers can be identified, including the epithelium (Ep), stroma and the Descemet's membrane (arrows). However, the corneal endothelium cannot be clearly resolved (arrow heads). In the image acquired using the IIS scheme (**Figure 3. 9b**), this thin single layer of squamous cells can be clearly identified by two interfaces (arrowheads in inset): the anterior interface between the Descemet's membrane (arrows) and the endothelium, and the posterior interface between the endothelium and the aqueous humor in the anterior chamber. These results demonstrated that the axial resolution restored by IIS scheme improved the capability of the ultrahigh resolution OCT.

Figure 3. 9 Cross-sectional images of swine cornea *ex vivo*. (a) A representative image acquired by the standard scheme where the endothelium layer (arrowheads) was not clearly resolved. (b) The corresponding image acquired by the IIS scheme where the endothelium layer was clearly resolved by

two interfaces (arrowheads). Ep: corneal epithelium; asterisks: interface between the corneal epithelium and the stroma; arrows: Descemet's membrane. Insets: 2X magnified view of the region in the box. Scale bars: 100 μm in tissue (refractive index = 1.38).

3.2.4 Discussion and Conclusion

IIS scheme is a novel platform of spectral-domain OCT that can potentially accommodate more reference beam manipulation techniques, in addition to spectral shaping. Multi-element camera lenses may be well corrected for optical aberrations in the spectrometer but multiple surface reflections cause the problem of low light throughput, especially for the very low-intensity optical signals back-scattered from the sample. In addition, the high-profile multi-element lenses and anti-reflection coatings are also very expensive. One can insert low-cost aberration correction optics in the reference path of the IIS to compensate the aberrations of the camera lens, without introducing additional loss to the sample signal. In most of OCT systems, single mode fibers are used so that the spot size at the line scan camera is proportional to the wavelength, leading to suboptimal roll-off performance at the long wavelengths in a linear-k spectrometer³¹. To mitigate this issue, one can use an endlessly single mode photonics crystal fiber³² in the reference arm to achieve a uniform spot size at the line scan camera over the entire spectral range. Although there will be coupling loss between single mode fiber and endlessly single mode photonics crystal fiber due to numerical aperture mismatch, only reference beam will be affected which can be tolerated within the optical power budget. However, there are some drawbacks of the IIS scheme. Firstly, it involves fiber pinhole coupling optics for the reference beam to overlap with the sample beam at the core of the spectrometer fiber terminal facet. This additional free space optics adds complexity and alignment difficulty with regard to the standard spectrometer. Secondly, it is difficult to tailor the spectral shape in this study which unavoidably is associated with the sidelobe problem.

In conclusion, we report a novel spectral-domain OCT design: interferometer-in-spectrometer scheme, which makes it possible to manipulate the reference beam for improved performances without compromising the detection sensitivity or significantly increasing the system cost. Our spectral shaping experiments validated the advantages

of the new scheme and improving axial resolution and ranging depth. Further studies are needed to full explore the possibilities opened up by this new scheme, such as aberration correction and improving the spot size of the spectrometer optics.

3.3 Summary

In this chapter, two OCT systems were presented: the μ OCT system with a standard scheme which was used for biological tissue imaging throughout Chapter 4-6 and the OCT system with a novel design in the spectrometer (i.e. IIS OCT) to mitigate limitations in ranging depth limited by the optical components in the standard system. With the validation of the improvement in axial resolution and ranging depth, this novel spectrometer design will be incorporated to the development of a ‘next-generation’ μ OCT system whose feasibility for biological tissue imaging will also be evaluated in different tissues.

Chapter 4

Optical Reflectance Contrast of Nuclei in Epithelium

4.1 Introduction and Background

Since OCT is regarded as a promising tool for cancer screening, it is important to understand the back-scattered signals from nuclei which carries important diagnostic information on cellular atypia such as increased nucleocytoplasmic ratio, poor cellular differentiation, and irregular cell arrangement.

The scattering characteristics of the nuclei in the stratified squamous epithelium (SSE) have been extensively investigated^{16,20,33-38}. However, excluding those studies that employed exogenous contrast agents such as acidic acid which increases the backscattering of the nuclei³⁹⁻⁴¹, there have been inconsistent reports on the scattering characteristics of cell nuclei, resulting in a standing confusion about whether the nuclei are ‘high scattering’ or ‘low scattering’ in squamous epithelial cells (keratinocytes). Specifically, both some reflectance confocal microscopic (RCM) and optical coherence microscopic (OCM) studies agreed that nuclei can be identified as dark areas or low scattering structures within the bright cytoplasm *in vivo*^{33,34,37}. Whereas, some other studies using RCM with <1 μm transverse resolution and OCM with $\sim 2 \mu\text{m}$ transverse resolution and $\sim 4 \mu\text{m}$ axial resolution reported that epithelial nuclei were bright or highly scattering relative to the cytoplasm^{35,36,42,43}.

In this thesis, using μOCT ¹⁹, we investigated the scattering characteristics of nuclei in the cultured human keratinocytes, rat epidermis, esophageal and cervical epithelium *ex vivo*, swine epidermis and oral mucosa *ex vivo*, human esophageal epithelium *ex vivo*, and human oral mucosa *in vivo*, respectively, and clarify that nuclei in both keratinized

and nonkeratinized stratified squamous epithelia are generally low scattering in the core. More importantly, in the nonkeratinized squamous epithelium such as those in the esophagus and oral mucosa, we could readily detect high scattering signals at the nucleocytoplasmic boundary sandwiching a dark nuclear core which manifest as bright dots within cells in the *en face* view. Our results suggest that those previously reported ‘high scattering’ or ‘bright’ nuclear signals in the esophageal and cervical epithelium were most likely from the nucleocytoplasmic boundary and the low scattering nuclear cores were missed possibly due to insufficient axial resolution^{35,36,42,43}.

4.2 Materials and Methods

Since the structural details of chromatin has yet to be well established for theoretical modeling using methods like finite-difference time-domain simulation, we experimentally assessed the nuclear back-scattering intensity with μ OCT system firstly in a simple condition, i.e. cultured epithelial cells and then in intact epithelial tissues.

4.2.1 Cultured Human Epidermal Cells

Primary human epidermal keratinocytes (ATCC PCS-200-010) were incubated with growth medium containing dermal cell basal medium (ATCC PCS-200-030) and keratinocyte growth kit (ATCC PCS-200-040) for 24 hours and then cultured with the growth kit (ATCC PCS-200-040) for another 3-4 days to achieve a density of 75-80%. Thereafter, the cells were seeded in Transwell inserts (Corning 3422) at a density of 5×10^4 cells/cm² and μ OCT imaging was performed when a cell density of ~50% was achieved. The Institutional Review Board (IRB) at Nanyang Technological University (NTU) approved the study (IRB 17/12/04).

4.2.2 Tissue Preparation and Histology

We evaluated the scattering intensity of nuclei in tissues from rat, swine and human. For rat studies *ex vivo*, 5 Sprague Dawley rats (female, 10-14 weeks) were sacrificed and organs including skin, esophagus and cervix were harvested. μ OCT imaging was immediately conducted and all the specimens were fixed with 10% neutral-buffered formalin (Leica Biosystems) for histological analysis. The study was approved by

Institutional Animal Care and Use Committee (IACUC) of NTU (ARF-SBS/NIE-A0312).

For swine studies *ex vivo*, we harvested the fresh skin and oral mucosa (floor of mouth) from 4 Landrace cross white pigs (female, 12 months) immediately after the cessation of vital signs. Specimens with a size of 1-2 cm $\times$ 2 cm were separately stored in serum-free Dulbecco's Modified Eagle's Medium (DMEM), before shipped on ice within 1 hour to the imaging site, where they were preserved in DMEM at 37°C ventilated with 95% oxygen and 5% carbon dioxide. μ OCT image acquisition of all specimens was completed within the next 2 hours. The specimens were then fixed for histology (RM2235, Leica Biosystems). The use of swine tissues was approved by the IACUC of National University of Singapore (R14-0797).

In addition, transmission electron microscopic (TEM) images of swine skin and oral mucosa were also acquired to explore the homogeneity of the nuclear inclusions. Specimens harvested from anesthetized cross Landrace swine (female, 12 months) were immediately immersed in 3% glutaraldehyde (pH = 7.4) at 4°C for pre-fixation. After being trimmed to $\sim 2 \times 1 \times 1$ mm, they were fixed with fresh 3% glutaraldehyde (pH = 7.4) for ~ 6 hours and post-fixed with 1% osmium tetroxide for ~ 2 hours at 4°C. Following routine TEM procedures, the specimens were embedded with Epon812. After the successful identification of intact mucosa by semithin sectioning, specimens were sectioned to 70-nm by ultramicrotome (LKB Ultratome V) and stained with uranyl acetate and lead citrate. The ultrastructural characteristics of epithelial cell nuclei from different organs were observed under transmission electron microscope (JEOL 1200EX) and recorded for analysis with Olympus Morada G2 camera. TEM images showed that the nuclear inclusions were relatively homogenous comparing to the cytoplasm in both keratinized and nonkeratinized SSEs (**Figure 4. 1**).

Figure 4.1 Representative TEMs of keratinocytes from swine keratinized skin (a) and nonkeratinized floor of mouth (b). Scale bars, 2 μ m.

For human esophagus studies *ex vivo*, μ OCT imaging was performed from the clean margin of 4 esophageal specimens. Following image acquisition, all the specimens were fixed overnight and subject to a standard histology processing protocol. The study was approved by the IRB at Renmin Hospital of Wuhan University (2017K-C053). In addition, we also recruited 2 human subjects and conducted μ OCT imaging of Lugol's positive human labial mucosa *in vivo*. The lower labial mucosa was in gentle contact with a food packing film, which coated the fused silica window of the flexible probe. 3D images were acquired within 17 seconds. 3% Lugol's solution of the lower labial mucosa was applied after μ OCT imaging. Written consents were obtained from volunteers before the experiment. The study was approved by the IRB at NTU (IRB-2016-10-015).

4.2.3 Normalized Back-scattering Intensity Measurement

The normalized back-scattering intensity is defined as the ratio between the back-scattered light intensity from the scatterers, i.e. nuclei under investigation and the back-reflected intensity from a gold-coated mirror. In μ OCT-based measurements, we firstly measured the back-reflected intensity from a gold-coated mirror, and then normalize all the back-scattered intensity to it. By doing so, the measured values can be compared between different tissues.

4.2.4 Statistical Analysis

The optical intensity was manually measured from 50 randomly selected locations within an area of 2×2 pixels and 8×8 pixels (width $\times$ height) at each location for nucleus

and cytoplasm, respectively. Here we normalize the optical intensity as the ratio between the optical power back-reflected or back-scattered from the intracellular components and that from a perfect reflector as mentioned above (reflectivity: 100%). All the numerical values are presented as mean $\pm$ standard deviation (SD) and $p < 0.05$ was regarded as a statistically significant difference. Independent-samples t-test was used to compare the intensity between the nuclei and cytoplasm of each specimen. One-way analysis of variation (ANOVA) was adopted to compare the intensity of cell nuclei among epithelial tissues and Bonferroni post hoc test was used for further comparisons if statistically significant difference was detected. All statistical analyses were conducted using SPSS software (IBM SPSS Statistics 23.0).

4.3 Experimental Results

4.3.1 Nuclei Scattering in Cultured Human Keratinocytes

μ OCT images of cultured keratinocytes provided cytologic information at a scale comparable to cytology (**Figure 4. 2**). From both cross-sectional (**Figure 4. 2 b1**) and *en face* views reconstructed from 3D OCT datasets (**Figure 4. 2 b2**), the nuclei demonstrated an intensity of $(0.13 \pm 0.07) \times 10^{-6}$, which was significantly lower than that of the cytoplasm $(1.16 \pm 0.55) \times 10^{-6}$ (t-test; $p < 0.001$). The low-scattering nuclei can be clearly identified including the two nuclei in a keratinocyte undergoing cell division (**Figure 4. 2 c&d**). These observations agree well with the previous results in cultured human glioblastoma cells⁴⁴.

Figure 4. 2 Optical intensity distribution in μ OCT images of cultured human keratinocytes. (a) A schematic of subcellular optical intensity distribution of cultured keratinocyte. (b) Representative μ OCT images of a keratinocyte viewed

from the cross-section (b1) and *en face* (b2) view: the optical intensity profile of the keratinocyte (blue dashed box) was derived from real-imaging data in (b2). (c) A keratinocyte undergoing cell division with two nuclei. (d) Representative cytology with a cell that has just completed nuclear division (arrow). NC: nucleocytoplasmic. Scale bars, 30 μm .

4.3.2 Nuclei in Keratinized Stratified Squamous Epithelium

We used tissues from albino rats and white swine to exclude the effect of melanin on cytoplasmic scattering³³. μOCT images acquired from the rat skin, esophagus and cervix and swine skin revealed well-developed keratinized layer (Figure 4. 3). Layered structures of mucosal wall and subcellular information of the epithelial cells (keratinocytes) were fully reproduced by μOCT (Figure 4. 3 a1-d1), matched well with the corresponding histology (Figure 4. 3 a2-d2). The nuclei of keratinocytes in all types of SSE were generally lower scattering than the cytoplasm (Figure 4. 3 a1-d1, yellow arrows).

Figure 4. 3 μOCT images of the orthokeratinized stratified squamous epithelium in rat and swine acquired *ex vivo*. (a1-d1) Cross-sectional μOCT images of the albino rat skin (inset is $2 \times$ zoom-in of selected area), esophagus,

cervix, and skin of white swine, respectively; (a2-d2) the corresponding histology with H&E staining. Yellow arrows indicate low-scattering nuclei. The contrast of all images was adjusted with imageJ for the optimal visualization of nucleus. KL: keratinized layer; EP: epithelium; LP: lamina propria. Scale bars, 50 μ m.

4.3.3 Nuclei in Nonkeratinized Stratified Squamous Epithelium

In the cross-sectional μ OCT images from swine floor of mouth (**Figure 4. 4**), we could frequently identify paired high-scattering signals at the nucleocytoplasmic boundary each sandwiching a low-scattering nuclear core in the middle and upper epithelial layers, which corresponded to the Periodic acid-Schiff-Diastase (PAS-D) positive cells with flattened nuclei based on the matched histology (**Figure 4. 4 d-f**, white arrows & arrowheads). In contrast, the high-scattering signals were less often visible in keratinocytes with PAS-D negative cytoplasm and roundish nuclei (**Figure 4. 4 a-c**).

Figure 4. 4 μ OCT images of swine floor-of-mouth acquired *ex vivo*. (a) A representative cross-sectional μ OCT image showing few high-scattering signals at the nucleocytoplasmic boundary (see Video S1, a); (b1) the corresponding histology with PAS staining and (b2) that with PAS-Diastase staining indicating cells with glycogen-negative cytoplasm and roundish nuclei; (c) *en face* μ OCT view of the upper third of the epithelium. (d) A representative cross-sectional μ OCT image presenting abundant high-scattering signals at the nucleocytoplasmic boundary; (e1) the corresponding histology with PAS staining and (e2) that with PAS-Diastase staining indicating cells with glycogen-positive cytoplasm and flattened nuclei; (f) *en face* μ OCT view of the upper third of the epithelium. Yellow arrows represents low-scattering nuclei; white arrows and arrowheads indicate high-scattering nucleocytoplasmic boundary. The contrast of all images was adjusted with imageJ for the optimal visualization of nucleus and nucleocytoplasmic boundary. Scale bars, 50 μ m.

The same nuclear scattering characteristics can be found in the cross-sectional images of the human esophageal epithelium *ex vivo* and the Lugol's-positive human labial mucosal epithelium *in vivo* (**Figure 4. 5a,c&f**, white arrows & arrowheads). These high-scattering signals demonstrated as bright dots in the *en face* views reconstructed from the 3D OCT datasets (**Figure 4. 5b&d**). Whereas, it is worth mentioning that cells in the nonkeratinized epithelium is actually keratinized but to a less degree of keratinization, and therefore, their nuclei may undergo pyknosis (condensation of chromatin) or fragmentation and the cytoplasmic organelles could disappear as they migrate to the epithelial surface. This phenomenon of the terminal maturation of keratinized cells could be captured by μ OCT in the human labial mucosa *in vivo* where cells presented bright irregular dots within empty ('dark') cytoplasm (**Figure 4. 5e**).

Figure 4. 5 μ OCT images of high-scattering signals at the nucleocytoplasmic boundary in the human esophageal and labial mucosal epithelium. (a) μ OCT cross-sectional and (b) *en face* images of the human oesophagus acquired *ex vivo*; lower-right inset in (a) is the corresponding histology. (c) μ OCT cross-sectional and (d) *en face* images of the human labial mucosa obtained *in vivo* showing high-scattering nucleocytoplasmic boundaries; lower-right inset in (c) is a schematic of scattering profile of squamous cells. Red dashed boxes in (a&c) are 2 $\times$ zoom view of selected areas; all white arrows and arrowheads indicate the high-scattering nucleocytoplasmic boundary. (e) μ OCT image of the most superficial layers of human labial mucosal epithelium *in vivo* and the blue arrows suggest pyknotic nuclei or cell debris encircled by bright cell borders. (f) A photograph of human labial mucosa with Lugol's staining marked by yellow arrowheads. The contrast of all images was adjusted with imageJ for the optimal visualization of nucleus and nucleocytoplasmic boundary. Scale bars, 50 μ m.

According to the images acquired by μ OCT, the axial range of the flattened nuclei was approximately 2.22-2.59 μ m, analogous to that in the corresponding histology ($\sim$ 2.38 μ m). Therefore, the upper and lower scattering signals at the nucleocytoplasmic boundaries may not be resolved in the previous studies due to the limited axial resolution ($\sim$ 4 μ m)^{35,36,42,43}, which, consequently, missed the fact that the nuclear cores were low

scattering. To validate our understanding, we degraded the axial resolution to $3.7\ \mu\text{m}$ in tissue algorithmically and it was clear that the low-scattering nuclear cores cannot be recognized on the cross-sectional images (**Figure 4. 6**).

Figure 4. 6 Cross-sectional μOCT images of human labial mucosa. (a) Low-scattering nuclear cores sandwiched by the high-scattering nucleocytoplasmic boundary are readily detectable by μOCT with an axial resolution of $\sim 1.3\ \mu\text{m}$ in tissue; (b) The low-scattering nuclear cores cannot be recognized leaving a vague bright dot of the nuclear region when the axial resolution is algorithmically degraded to $3.7\ \mu\text{m}$ in tissue. Scale bars, $50\ \mu\text{m}$.

4.3.4 Quantitative Analysis of Nuclei Optical Intensity

Finally, we measured the scattering intensity of nuclei from some of SSE investigated and normalized the intensity of nuclear cores to a perfect reflector. It can be seen that nuclei were consistently low scattering with respect to their cytoplasm (**Table 4. 1**; Independent-samples t-test). We also compared the optical intensity of nuclei among epithelial tissues, which demonstrated that the nuclei in rat and swine epidermis were statistically higher than those in the rest epithelia, and further Bonferroni post hoc tests showed that, excluding the epidermis, there were no statistically significant differences in nuclear intensity among the rat esophagus and cervix, swine floor of mouth (PAS-negative) and human labial mucosa (One-way ANOVA, $F=47.60$, $p < 0.001$; **Figure 4. 7**).

Table 4. 1 Optical intensity of the nuclear core and cytoplasm in different mammalian SSE[†]

SSE Type	nuclear core (mean $\pm$ SD) $\times 10^{-6}$	cytoplasm (mean $\pm$ SD) $\times 10^{-6}$	$P^{\S}$
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Rat skin	0.45 ± 0.22	9.10 ± 5.83	$< 0.001^*$
Rat esophagus	0.14 ± 0.06	1.11 ± 0.30	$< 0.001^*$
Rat cervix	0.16 ± 0.10	2.23 ± 1.63	$< 0.001^*$
Swine skin	0.38 ± 0.17	5.19 ± 1.91	$< 0.001^*$
Swine floor-of-mouth	0.19 ± 0.13	1.54 ± 0.61	$< 0.001^*$
Human labial mucosa	0.15 ± 0.07	1.38 ± 0.49	$< 0.001^*$

[†]Swine floor-of-mouth (PAS +) and human esophagus were not included due to insufficient data;

[§]Independent-samples t-test;

*The mean difference is statistically significant at the 0.05 level.

Figure 4. 7 A boxplot displays the optical intensity of nuclear cores among mammalian SSE.

4.4 Discussion and Conclusion

This study establishes that cell nuclei of SSE in mammals can be visualized by their negative contrast against the cytoplasm or the high scattering nucleocytoplasmic boundary in optical coherence tomography images at 800 nm. Our study clarifies the discrepancy among the previous observations and provides a consistent and comprehensive interpretation of the optical characteristics of nuclei in SSE.

While Aguirre. et al mentioned that the nuclei in the human epidermis were not readily visible using OCM³⁶, several RCM and OCM studies reported the visualization of ‘dark’ nuclei in human skin, where melanin provides the strong contrast for the

cytoplasm^{33,34,37}. To disclose the native scattering property of nuclei and the nucleocytoplasmic contrast in the intact epidermis, tissues from albino animals and white swine were used in this study. In contrast to images from the pigmented skin that the basal layer demonstrates the brightest intensity due to melanin accumulation³³, our results showed that the basal layer in the non-pigmented SSE presented relatively lower intensity than the middle and upper layers. We found that the nuclei of differentiated cells remained to be low scattering relative to the cytoplasm when melanin was absent, agreed with previous studies in rat bladder and cultured human glioblastoma cells⁴⁴⁻⁴⁶. Note that the scattering signals from the cytoplasm may be too speckly to clearly delineate the negative-intensity areas of nuclei in individual OCT/OCM images, and this speckle issue could be resolved by averaging multiple time-lapsed frames or consecutive *en face* frames^{45,46}.

Nuclear flattening and glycogen accumulation are hallmarks of cell maturation in the nonkeratinized SSE such as those in the esophagus and cervix. The high scattering signals were more commonly found in the upper half epithelial layers where an epithelial cell tends to have a more flattened nucleus due to maturation, and this morphological change may be associated with the signals at the nucleocytoplasmic boundary. The current study also suggests that the nucleocytoplasmic high-scattering signals may co-exist with the glycogen accumulation in the cytoplasm (PAS+). Therefore, the paired, high-scattering signals at the nucleocytoplasmic boundary could be used to evaluate cell maturation since the normal maturation processes are disrupted in neoplasia or cancers, including nuclei flattening and glycogen accumulation. It is possible that maturation associated cell events such as glycogen accumulation might have changed the refractive index difference between the cytoplasm and nuclei, as cell nuclei normally have lower refractive index and mass density than cytoplasm^{47,48}. However, the cell physiology is rather complicated and a serial of signal pathways may be activated or repressed responding to the signals for glycogen accumulation, nuclei flattening and other cell maturation related events such as alterations in chromatin density within nuclei. Since intracellular glucose could also alter the transcription program of the cells^{49,50}, it is possible that the refractive index of the nucleus may be affected owing to the varied level of gene expression. It is the limitation of the current study that the refractive index changes of the cytoplasm and the nucleus during maturation were not studied. Further investigations will focus on analyzing the

refractive index of cytoplasm and nucleus of glycogen-rich keratinocytes and exploring the correlation between the OCT signals and the cytologic changes.

This study clarifies that the origin of the high-scattering signals is the nucleocytoplasmic boundary instead of the nuclear core, which lays the basis for the improved understanding that these high scattering signals may indicate cell flattening and can be used as an imaging marker of cell maturation. To demonstrate the benefit of this new imaging marker, validation studies will be conducted on animal models and human samples with epithelial neoplasia against the clinical gold standard. Another unresolved question is why the nuclear cores are low scattering. We have conducted a preliminary study on the ultrastructural characteristics of the keratinocytes in SSEs with transmission electron microscopy. In both keratinized and nonkeratinized SSE, the nuclear inclusions were homogenously distributed relative to those in the cytoplasm (**Figure 4. 1**), suggestive of much less refractive index heterogeneity within the nucleus than the cytoplasm.

We also compared the intensity of nuclear cores among tissues which indicated that nuclear optical intensity was mostly similar across types of SSE except for the epidermis. Since we know that the influence of speckles increases with the reflectivity of the sample, we attribute the measured high nuclear intensity in epidermis to speckles from the cytoplasm or the nucleocytoplasmic boundary, which might have been included in the manually-determined areas of nuclei due to the resolution limitation. In addition, other imaging artifacts such as attenuation of light by epithelial layers over the measured cell, autocorrelation artifacts from the highly reflective or scattering keratinized layer, lamina propria, or cytoplasm might also have altered the measured values. It was also worth mentioning that some OCT images could couple with background noises and artifacts, owing to autocorrelation or tissue surface reflection. Therefore, during the measurement of optical backscattering intensity, we have avoided images and regions affected by background artifacts.

In conclusion, this study establishes the negative contrast of nuclei in the mammalian SSE by use of μ OCT operating at 800 nm in both small and large animals *ex vivo*, and human labial mucosa *in vivo*. We unify most of the previous inconsistent reports on the nuclear scattering characteristics which may contribute to the establishment of a consensus on nuclear scattering properties and new OCT diagnostic criteria based on

the subcellular reflectance contrasts. Future works on elucidating the biological background underlying the subcellular scattering contrasts may open up a new avenue towards ‘virtual histology’ over a large mucosal area *in vivo*.

Chapter 5

Optical Reflectance Contrast of Cytoplasm in Epithelium

5.1 Introduction and Background

Epithelial cells are typically specialized with variations in their intracellular inclusions, suited to the particular task in a specific organ⁵¹. While cell nuclei have a quasi-uniform distribution of its compositions and consistently present low back-scattering in the core^{19,44,52}, the cytoplasm is the predominant site reflecting the specialization-mediated intracellular variations and corresponding alterations in their back-scattered intensity. Saidi *et al.* used Mie theory to approximate skin tissue scattering but by assuming cells are homogeneous spheres of a single size⁵³. Dunn and Drezek *et al.* used the FDTD method to model light scattering from cells containing multiple organelles of arbitrary shape^{54,55}. Their studies suggest that small organelles whose size is comparable to the wavelength of light play a more important role than the nucleus in scattering from a cell^{54,55}, such as mitochondria⁵⁶ and melanin granules³³.

However, most of the previous efforts were focused on the scattering contributions from micrometer-scale quasi-spherical scatterers, and little is understood on the back-scattering (reflectance) from nanometer scale scatterers, in particular, clustered mucin granules and packed keratin filaments, and microvilli. Furthermore, the effects of their biological variations on the back-scattered intensity of cells under different physiological states are largely unknown. In this thesis, we investigated the micro- and ultrastructural bases underlying the cytoplasmic optical reflectance contrast in a wide variety of specialized epithelia both theoretically using the FDTD method and experimentally using a cellular-resolution OCT (μ OCT). We further validate the

feasibility of the improved understandings on the optical reflectance contrast of epithelial cells for real-time characterization of epithelial cancers and their precursors.

5.2 Materials and Methods

5.2.1 Modelling of scatterers using FDTD Method

Modelling of clustered or packed nanometer scale scatterers was conducted using FDTD method with Lumerical software (Lumerical FDTD Solutions, Vancouver, Canada). Simulating the reflectance imaging system of μ OCT was conducted based on the scalar diffraction theory and the optical parameters of the sample arm optics, which are described in details in **Chapter 2**. The spatial and optical parameters of models were obtained from TEM images and published data, and are provided in **Table 5. 1-Table 5. 3** for mucin granules in mucous cells (**Table 5. 1**), microvilli in enterocytes (**Table 5. 2**), and keratin filament in keratinocytes (**Table 5. 3**), respectively.

Table 5. 1 Parameters of mucin granules in FDTD modelling

Optical parameters	Value	
Sources	Gaussian wave	
Collection numerical aperture	0.2 (1% power level)	
Wavelength (nm)	700-950 with a 6.25 increment	
Mesh grid (nm)	30	
Polarization	Unpolarized	
Refractive index of cytosol	1.34 (from ref. 57)	
Scatterers: Mucin granules	Stomach	
Refractive index	1.38 (from ref. 58)	1.41 (from ref. 58)
Geometry	Sphere	Sphere
Diameter (nm)	202 (137-265) ^a	202 (137-265) ^a
Density (numbers/ μm^3)	18.1 ^b	18.1 ^b
Focal volume ($\mu\text{m} (x) \times \mu\text{m} (y) \times \mu\text{m} (z)$)	$2 \times 2 \times 2$	$2 \times 2 \times 2$
Normalized back-scattered intensity	6.7×10^{-6}	1.8×10^{-5}

^aData acquired from TEM images of swine stomach (n=50).

^bDetermined by the center-center inter-granules distance of ~380 nm measured from

TEM images of swine stomach (n=50).

Table 5. 2 Parameters of microvilli in FDTD modelling

Optical parameters	Value			
Sources	Gaussian wave			
Collection numerical aperture	0.2 (1% power level)			
Wavelength (nm)	700-950 with a 6.25 increment			
Mesh grid (nm)	10			
Polarization	Unpolarized			
Refractive index of cytosol	1.34 (from ref. 57)			
Scatterers: Microvilli	Small intestine		Colon	
Refractive index	1.38 (from ref. 58)	1.41 (from ref. 58)	1.38 (from ref. 58)	1.41 (from ref. 58)
Geometry	Cylinder		Cylinder	
Diameter (nm)	100 (from ref. 59)		90	
Height (nm)	1010-1172 (from ref. 59) ^a		140 – 510 ^b	
Density (numbers/ μm^2)	81.8 (from ref. 59)		33.0 ^c	
Focal volume ($\mu\text{m} (x) \times \mu\text{m} (y) \times \mu\text{m} (z)$)	$2 \times 2 \times 2$		$2 \times 2 \times 2$	
Normalized back-scattered intensity	2.5×10^{-5}	7.4×10^{-5}	0.39×10^{-5}	1.2×10^{-5}

^a Measured over 2- μm lateral field of view.

^b Data acquired from TEM images of swine colon (n=50) over 2- μm lateral field of view.

^c Determined by the center-center inter-microvillus distance of 140-340 nm (n=50).

Table 5. 3 Parameters of keratinocyte filament in FDTD modelling

Optical parameters	Value	
Sources	Gaussian wave	
Collection numerical aperture	0.2 (1% power level)	
Wavelength (nm)	700-950 with a 6.25 increment	
Mesh grid (nm)	2	
Polarization	Unpolarized	
Refractive index of cytosol	1.34 (from ref. 57)	
Scatterers: Keratin filament	Tonofilament bundles	Dispersed tonofilaments
Packing state	parallel and dense packing	parallel and loose packing

Orientation	Perpendicular to the light propagation	Perpendicular to the light propagation
Refractive index	1.40 (from ref. 57)	1.40 (from ref. 57)
Geometry	Cylinder	Cylinder
Diameter (nm)	8	8
Density (numbers/ μm^2)	5972 ^a	4276 ^b
Structure volume ($\mu\text{m} (x) \times \mu\text{m} (y) \times \mu\text{m} (z)$)	$0.5 \times 0.5 \times 0.5$	$0.5 \times 0.5 \times 0.5$
Focal volume ($\mu\text{m} (x) \times \mu\text{m} (y) \times \mu\text{m} (z)$)	$2 \times 2 \times 2$	$2 \times 2 \times 2$
Number of structures per focal volume	14 ^c	14 ^c
Normalized back-scattered intensity	5.46×10^{-6}	3.08×10^{-6}

^{a,b}Determined by the center-center inter-tonofilament distance of 16 nm and 24 nm.

^cEstimated based on TEM images of swine epidermis.

5.2.2 Normalized Back-scattering Intensity Measurement

The normalized back-scattered intensity is defined as the ratio between the back-scattered light intensity from the scatterers under investigation and the back-reflected intensity from a gold-coated mirror. In μOCT -based measurements, we firstly measured and the averaged back-reflected intensity from cytoplasm within an area of 10×8 pixels (width $\times$ height) and the back-reflected intensity from a gold-coated mirror. Thereafter, we calculated the normalized back-scattered intensity. By doing so, the measured values are independent of the imaging system, and therefore, can be compared between different experiments and with the simulated values.

5.2.3 Tissue Preparation and Histology

Skin, floor of mouth, esophagus, stomach, small intestine and colon from 4 cross Landrace white pigs (female, 12 months) were harvested immediately after the cessation of vital signs. They ($1\text{-}2\text{ cm} \times 2\text{ cm}$) were separately stored in serum-free Dulbecco's Modified Eagle's Medium (DMEM) (Gibco). Specimens were shipped on ice within 1 hour to the imaging site, where they were preserved in DMEM at 37°C ventilated with 95% oxygen and 5% carbon dioxide. μOCT image acquisition of all specimens was

completed within the next 1 hours. Following μ OCT imaging, the specimens were submitted for routine histological procedures, sectioned into 5- μ m sections (RM2235, Leica Biosystems), and stained with hematoxylin-eosin (H&E) (Leica Biosystems). To evaluate the histochemical property of mucins between mucus-secreting cells, we also performed periodic acid-schiff (PAS) and alcian blue (AB) staining of sections from stomach and colon (Leica Biosystems). The use of swine tissues was approved by the Institutional Animal Care and Use Committee (IACUC) of National University of Singapore (R14-0797). μ OCT imaging of above-mentioned tissues except for the oral mucosa were conducted in another 2 white micropigs (female, 12 months) *in vivo*. We anesthetized the animals and surgically opened the lumen to access the epithelial surface of internal organs. Epithelial surface of each organs was washed with phosphate buffered saline (pH = 7.4; Gibco) prior to μ OCT image acquisition. The study was approved by the IACUC of PWG Genetics Pte Ltd (PN16076).

We also acquired μ OCT images from mouse esophagus and human specimens with epithelial precancerous lesions. C57BL/6 mice (female, 6 weeks) treated with 4-nitroquinoline 1-oxide (4NQO) at 100 μ g/ml in the drinking water were used to develop esophageal cancer⁶⁰. The mice were sacrificed every 2 weeks starting in week 14 and the esophagus was harvested, opened longitudinally. μ OCT images were sequentially acquired from 6-10 locations from the proximal to the distal end. Regions with visible tumors or intratumor vessels were excluded. Following OCT imaging, specimens were fixed for pathological analysis. OCT datasets with landmarks such as blood vessels and epithelial rete pegs that well-matched with the corresponding histopathology were selected for analysis. We acquired approval from the IACUC of NTU (ARF-SBS/NIE-A0319).

Human endoscopically resected specimens were also used for μ OCT image acquisition. Four esophageal specimens with pathologically confirmed severe dysplasia or superficial cancer were used and μ OCT images were acquired from both the normal and abnormal area. In stomach, 3 specimens were included including 2 without epithelial lesions and 1 with intestinal metaplasia (IM). In the one with IM, μ OCT images were obtained from the smaller depressed lesion which were confirmed with monofocal IM pathologically. Eleven colorectal polyps including 6 non-adenomatous polyps and 5 adenomatous polyps were included, and μ OCT images were acquired from 2-4 random

locations for each specimen. Following image acquisition, all the specimens were fixed overnight and subject to a standard surficial specimen processing protocol. The specimen was sectioned at the interval of 2 mm in width and embedded in its entirety for histopathological diagnosis. Written informed consent was obtained from participants prior to the study and the use of human tissues was approved by the IRB at Renmin Hospital of Wuhan University (2017K-C053).

5.2.4 Statistical Analysis

A quantitative analysis of the cytoplasmic optical intensity were conducted among cells in normal swine epithelial tissues. We manually measured the averaging intensity from 50 randomly selected locations within an area of 10×8 pixels (width $\times$ height) for each location. The data for each epithelial type were from the 1024 images of one three-dimensional (3D) OCT dataset. Independent-samples t-test and One-way analysis of variation (ANOVA) was adopted to compare optical intensity inbetween mucus-secreting cells and among keratinocytes at different level of maturation, respectively. Further Bonferroni post hoc test was performed if statistical differences were detected. A $p < 0.05$ was regarded as a statistically significant difference. All numerical values were presented as mean $\pm$ standard error (SE). All statistical analyses were conducted using SPSS software (IBM SPSS Statistics 23.0).

5.3 FDTD Simulation and Experimental Results

We firstly developed back-scattering models of mucin granules, microvilli, and keratin filaments using the FDTD method according to transmission electron microscopic (TEM) images and previously published data (**Table 5. 1-Table 5. 3**). The results showed that these clustered or packed nanometer-scale scatterers may contribute significantly or even dominantly to cell back-scattering, depending on their clustering or packing states. The corresponding experimental realizations were carried out using μ OCT on intact mammalian epithelia including simple columnar epithelia (SCE) from swine stomach, colon and small intestine, and stratified squamous epithelia (SSE) from swine skin (orthokeratinized), esophagus (parakeratinized) and floor of mouth (nonkeratinized), respectively⁵². A summary of the reflectance contrast and the scatterers' features of epithelial cytoplasm is presented in **Figure 5. 1**. In this thesis, we

on one hand validated our theoretical predictions by correlating the cytoplasmic reflectance contrasts with the micro- or ultrastructural features of the above mentioned cytoplasmic inclusions pertaining to the physiological states of specialized epithelial cells; on the other, our theoretical analysis provide meaningful explanations of the mechanisms underlying the observed back-scattering phenomena. Thereafter, we conducted a preliminary study to evaluate the feasibility of the new understandings on the image contrast for the interpretation of back-scattering features in precancerous lesions using specimens from the mice esophageal and human gastrointestinal tracts.

Figure 5. 1 Summary of cytoplasmic reflectance features of specialized epithelial cells. (a-c) Boxplots of cytoplasmic optical intensity of cells in swine epithelial tissues: (a) comparison between foveolar cells and goblet cells; (b) comparison of epidermal keratinocytes from basal, prickle and granular layer, respectively; (c) comparison of esophageal keratinocytes from basal, prickle and granular layer. Data are mean $\pm$ standard error. (d) Schematics of optical reflectance contrast and its ultrastructural feature of epithelial cells under different physiological states.

5.3.1 Scatterers of Mucus Cells in Gastric and Colonic Epithelium

5.3.1.1 Reflectance contrast of mucin-secreting cells in normal stomach and colon

Both gastric foveolar cells and colonic goblet cells are highly polarized mucin-secreting cells which are important components of the gastrointestinal tract by producing the uttermost defense line of the **epithelia**. Although both cells demonstrated a similar ‘empty’ appearance of the apical cytoplasm in H&E stained sections and have the same histochemical properties of mucins (**Figure 5. 2 c1-c3 & Figure 5. 3 c1-c3**), their ultrastructural make-ups are very different: the mucin cups of foveolar cells were heterogeneously filled with nanometer-scale, discrete, electron-dense granules circled by relatively low-electron-density surroundings; in contrast, those of goblet cells were homogenous and full of fused, low-electron-density mucin granules (**Figure 5. 2d & Figure 5. 3d**). We modelled mucin granules in foveolar cells as nanoscale spheres suspended in the cytosol. The refractive index data of mucin granules is not available so the refractive index range of cell organelles was used as the best estimate⁶¹. The simulated normalized back-scattered intensity ranges from $(6.7-18) \times 10^{-6}$, which was one of the brightest intracellular structures investigated in this study (**Table 5. 1**). We predicted that the back-scattering intensity from mucin granules of goblet cells were insignificant since there was no noticeable electron density change in the TEM images and thus simulation was not conducted.

Figure 5. 2 Subcellular reflectance contrast of gastric foveolar cells. (a,b) Representative μ OCT cross-sectional (a) and *en face* (b) images acquired *ex vivo* present bright apical portion of foveolar cells (white arrows); the inset in (a) is a schematic of the back-scattering profile of a foveolar cell; the red dashed box in (b) is zoomed-in by 3 $\times$. (c1-c3) Corresponding histology with H&E, PAS and AB staining, respectively; (d) TEM image at a magnification of 5,000 $\times$ and the red dashed box is zoomed-in by 4 $\times$ showing mucin granules (MGs) of foveolar cells. N: nucleus. Scale bars for μ OCT and routine histological images, 50 μ m; scale bars for TEMs, 5 μ m.

Figure 5. 3 Subcellular reflectance contrast of colonic goblet cells. (a,b) Representative μ OCT cross-sectional (a) and *en face* (b) images obtained *ex vivo* demonstrate low-reflectance apical portion of goblet cells (yellow arrows); the inset in (a) is a schematic of the back-scattering profile of a goblet cell. (c1-c3) Corresponding histology with H&E, PAS and AB staining, respectively; (d) TEM image at a magnification of 5,000 $\times$ and the red dashed box is zoomed-in by 4 $\times$ showing mucin granules (MGs) of goblet cells. N: nucleus. Scale bars for μ OCT and routine histological images, 50 μ m; scale bars for TEMs, 5 μ m.

In μ OCT images, the asymmetric optical contrasts of mucin-secreting cells well reproduced the polarization feature of the cells (**Figure 5. 2a & Figure 5. 3a**). In agreement with our simulation results, foveolar cells demonstrated a high reflectance contrast in the apical cytoplasm relative to the low intensity in the basal portion where nuclei resided (**Figure 5. 2a,b**, white arrows). These bright signals lining gastric mucosa signified well-polarized foveolar cells, a finding that has unfortunately long been overlooked^{18,62} or misunderstood^{35,41}. In contrast, goblet cells could be identified by the low reflectance contrast of their barrel-shaped apical cytoplasm relative to adjacent enterocytes whose apical portion was relatively bright (**Figure 5. 3a,b**, yellow arrows).

These back-scattering features of goblet cells agreed with previous reports in human and rat^{35,63}. The normalized back-scattered intensity of the mucin cups of foveolar cells was measured to be $(2.74 \pm 0.09) \times 10^{-6}$, which was significantly higher than that of goblet cells $(0.28 \pm 0.02) \times 10^{-6}$ (**Figure 5. 1a**; independent-samples t-test; $p < 0.01$), which agrees well with their ultrastructural differences (**Figure 5. 2d & Figure 5. 3d**) and the theoretical predictions.

5.3.1.2 Reflectance contrast abnormalities in stomach with intestinal metaplasia

In normal gastric mucosa, human foveolar cells which contains exclusively of neutral mucins (only PAS positive) presented high scattering at the apical cytoplasm relative to the basal portion (**Figure 5. 4 a1-b3**, white arrows), allowing the delineation of the mucosal wrinkles and the opening of gastric pits. By contrast, in the region with pathologically diagnosed IM, metaplasia could be readily differentiated from the normal gastric mucosa as cell clusters were devoid of the characteristic high-intensity mucin cups of foveolar cells (**Figure 5. 4 b1-b3**, red arrowheads). According to the anatomically matched histology, those cells probably corresponded to the specialized columnar cells whose apical cytoplasm appeared purple with PAS- alcian blue (PAS-AB) staining (**Figure 5. 4 b4**). Interspersed between those columnar cells, goblet cells could be detected by their barrel- or cylinder- shaped low-scattering apical cytoplasm (**Figure 5. 4 b1,b2**, yellow arrows) which were stained blue with PAS-AB staining (**Figure 5. 4 b4**).

Figure 5. 4 Subcellular reflectance contrast of human gastric mucosa with intestinal metaplasia imaged by μ OCT *ex vivo*. (a1, a2) OCT cross-sectional (a1) and *en face* (a2) images of a specimen with normal mucosa and the white arrows indicates foveolar cells. (b1-b4) OCT cross-sectional (b1,b2) and *en face* images (b3) of another specimen from the region with intestinal metaplasia (red arrowheads): white arrows in (b1-b3) indicate foveolar cells and yellow arrows in (b1,b2) represent ‘ectopic’ goblet cells featured with low-scattering apical cytoplasm; corresponding pathology (b4) with PAS-AB mucin staining to highlight metaplastic area (red arrowheads). Scale bars, 50 μ m.

5.3.1.3 Reflectance contrast abnormalities in colon with neoplastic polyps

In non-neoplastic polyps (**Figure 5. 5 a2**), goblet cells could be readily recognized by their low-intensity apical cytoplasm relative to the enterocytes (**Figure 5. 5 a1,a3**, yellow arrows). Crypts were typically oval with uniform shape and size manifesting a ‘honeycomb’ pattern and their pits were commonly in small oval shape in the *en face*

view reconstructed from the 3D μ OCT dataset (**Figure 5. 5 a3**). Whereas, in μ OCT images obtained from polyps with pathologically confirmed adenoma (**Figure 5. 5 b2**), goblet cells were less often visible in comparison with those obtained from non-neoplastic polyps (**Figure 5. 5 b1,b3**) and dysplastic cells featured with cigar-shaped pseudostratified nuclei pathologically could be recognized by the elongated, irregularly arranged low-reflectance nuclei (**Figure 5. 5 b1**, red arrows). Crypts were laterally extended or distorted resulting in large tubular or gyrus-like pits in the *en face* view (**Figure 5. 5 b3**). All these μ OCT observations matched well with corresponding histology.

Figure 5. 5 Subcellular optical reflectance contrast of human colorectal polyps imaged by μ OCT *ex vivo*. (a1-a3) Representative μ OCT cross-sectional image (a1), corresponding histology (a2) and *en face* μ OCT view (a3) of a hyperplastic polyp. (b1-b3) Representative μ OCT cross-sectional image (b1),

corresponding histology (b2) and *en face* μ OCT view (b3) of an adenomatous polyp. Yellow arrows represent goblet cells and red arrows indicate low-scattering nuclei. Scale bars, 50 μ m.

5.3.2 Scatterers of Enterocytes in the Small Intestinal Epithelium

Enterocytes are epithelial cells fulfilling the function of absorption, whose apical surfaces are characterized by the presence of microvilli driven by polarization. The tips of microvilli are tightly and evenly packed, and the surface formed by their tips is equivalent to a high grade optical surface (**Table 5. 2**). We modelled microvilli as nanocylinders and used the previously reported refractive index range of cell organelles as the best estimate (**Table 5. 1**)⁶¹. FDTD simulation results show that the normalized back-scattered intensity of brush border ($(2.5-7.4) \times 10^{-5}$) was at least one order of magnitude higher than most of the cytoplasmic components in this study (**Figure 5. 6a**; **Table 5. 1-Table 5. 3** and **Figure 5. 1**). According to this prediction, a bright line in μ OCT images at the epithelial surface of the small intestine was expected. We also simulated microvilli in the colon epithelium, and the results show that the decrease in the packing density leads to a dramatic decrease in the back-scattered intensity ($(3.9-12) \times 10^{-6}$) (**Figure 5. 6b**; **Table 5. 2**).

Figure 5. 6 Subcellular reflectance contrast of enterocyte microvilli. (a,b)

Numerical models of microvilli using FDTD simulation of from small intestine (a) and colon (b). (c) A representative μ OCT cross-sectional image acquired *in vivo* showing a high-scattering fine structure overlying the apical surface of enterocytes (white arrows), suggestive of brush border. The lower-right inset is a schematic of the scattering profile of an enterocyte. (d) Corresponding histology with H&E staining and the red dashed box is zoomed in by 3x with PAS staining that highlights brush border (green arrows) and goblet cell (red arrows). Scale bars for original images, 50 μ m; scale bar for inset in (d), 10 μ m.

In μ OCT images of the small intestine, we did identify high-reflectance contour-like signals lining the luminal epithelial cell surface *in vivo* (**Figure 5. 6c**, white arrows), which could seldom be observed in the stomach and colon. The height of the structure was $1.35 \pm 0.07 \mu\text{m}$ away from the epithelial surface in μ OCT images (refractive index = 1.33; n=20), similar to that in histology ($1.23 \pm 0.04 \mu\text{m}$, n=20). The measured normalized back-scattered intensity was $(1.26 \pm 0.10) \times 10^{-5}$ (n = 100) agree well with the simulation result. Such a strong back-scattered signal can only be from the brush border (**Figure 5. 6d**, inset, green arrows) and the measured value.

5.3.3 Scatterers of Keratinocytes in the Stratified Squamous Epithelium

In μ OCT images of the squamocolumnar junction of albino rat cervix *in vivo*, keratinocytes presented high-scattering cytoplasm, in striking contrast with the adjacent low-scattering columnar cells (**Figure 5. 7 a1,a2**). This observations indicated that the high cytoplasmic intensity was correlated with keratinization, which could also be supported by the evidences from albino rat vagina *ex vivo* whose epithelium could alternate between mucification and keratinization provoked by progesterone and estrogen respectively during estrous cycle (**Figure 5. 7 b1-d2**).

Figure 5. 7 Subcellular reflectance contrast of albino rat cervix *in vivo* (a1, a2) and vagina *ex vivo* (b1-d2). (a1, b1, c1, and d1) Representative μ OCT cross-sectional images and (a2, b2, c2, and d2) corresponding histology with H&E staining. (a1, a2) Cervical epithelium at squamocolumnar junction showed low-scattering simple columnar epithelium (SCE; red arrowheads) and high-scattering stratified squamous epithelium (SSE; blue arrowheads). (b1, b2) At diestrus, vaginal epithelial cells were full of mucin granules demonstrating middle-low optical intensity. (c1, c2) As it approached proestrus, keratinocytes replaced mucus cells, manifesting high cytoplasmic intensity. (d1, d2) At estrus, epithelial keratinization reached its maximum, characterized by a well-developed keratinized layer and keratinocytes present bright cytoplasm. All yellow arrows represent dark nuclei and red arrows indicate bright cell borders. ME: mucified epithelium; KL: keratinized layer; LP: lamina propria. Scale bars, 50 μ m.

5.3.3.1 Keratin filaments of keratinocytes in the normal skin, esophagus, oral mucosa

At the ultrastructural level, the clustering or packing states of keratin filaments vary dramatically between keratinocytes of different degree of keratinization (**Figure 5. 8- Figure 5. 10**). Generally, two types of packing states could be observed: 1. closely

packed and well aligned keratin filaments in highly clustered bundles, which are also known as tonofibrils, such as those in the prickly layer and the granular layer of the orthokeratinized epidermis (**Figure 5. 8d,e**), and the prickly layer of the parakeratinized esophageal epithelium (**Figure 5. 9d**) and nonkeratinized oral mucosa (**Figure 5. 10d**); 2. Much less aggregated, loosely dispersed individual filament such as those in the intermediate layers of the parakeratinized esophageal epithelium (**Figure 5. 9e**), and nonkeratinized oral mucosa (**Figure 5. 10e**, yellow arrows) in swine. In particular, in the TEM of swine esophageal epithelium, we could see clearly the variation of the packing state as the cell evolved from the prickly layer to the intermediate layer (**Figure 5. 9d-e**). We developed FDTD models of the two packing states of keratin filaments and the results show that the normalized backscattered intensity of tonofilaments in closely packed state was 5.46×10^{-6} , which was larger than those in the loosely dispersed state 3.08×10^{-6} (**Table 5. 3**).

Figure 5. 8 Subcellular reflectance contrast of keratinocytes in the swine epidermis (orthokeratinized epithelium). (a) A representative *in vivo* μ OCT cross-sectional image; (b) corresponding histology with H&E staining; (c-f) representative TEMs of cells from the basal layer (c), prickly layer (d), granular layer (e) and keratinized layer (zoomed-in view of rectangular area in f) at 50,000 $\times$ magnification, respectively. All green arrows indicate keratin filament bundles. N: nucleus; SC: stratum corneum. Scale bars for μ OCT and H&E

stained images, 50 μm ; scale bars for TEMs, 0.5 μm .

In μOCT images of swine SSEs with different degree of keratinization, we found that the cytoplasmic optical intensity of keratinocytes was indeed associated with the packing states of intracellular keratin filaments: cells presenting bright cytoplasm had filaments that were closely packed into bundles; those demonstrating relatively low intensity contained filaments that were loosely dispersed within cytoplasm (**Figure 5. 8-Figure 5. 10**).

Figure 5. 9 Subcellular reflectance contrast of keratinocytes in the swine esophagus (parakeratinized epithelium). (a) A representative *in vivo* μOCT image; (b) corresponding histology with H&E staining; (c-f) representative TEMs of cells from the basal layer (c), prickle layer (d), intermediate layer (e) and keratinized layer (zoomed-in view of rectangular area in f) at 50,000 $\times$ magnification, respectively. Green and orange arrows indicate keratin filament bundles and individual keratin filament, respectively; red arrows indicate cell borders. N: nucleus. Scale bars for μOCT and H&E stained images, 50 μm ; scale bars for TEMs, 0.5 μm .

Those cells rich in densely packed tonofilaments presented much higher normalized back-scattered intensity than those with loosely dispersed tonofilaments (**Figure 5.**

1b,c). Specifically, in the parakeratinized swine esophageal epithelium, keratinocytes in the prickly layer demonstrated a cytoplasmic intensity of $(3.34 \pm 0.19) \times 10^{-6}$, which is significantly higher than that of the intermediate layer $((2.05 \pm 0.13) \times 10^{-6})$ (**Figure 5. 1c**; one-way ANOVA, $p < 0.01$). These measured data agree with the variations in the packing state of the filaments (**Figure 5. 9d-e**) and the FDTD predictions (**Table 5. 3**). Note that in swine epidermis cells at the granular layer was brighter than that at the prickly layer (**Figure 5. 1b**; one-way ANOVA, $p < 0.01$), which is known due to the densely packed keratohyalin granules⁶⁴.

Figure 5. 10 Subcellular reflectance contrast of keratinocytes in the swine oral mucosa (nonkeratinized epithelium). (a) A representative *in vivo* μ OCT cross-sectional image; (b) corresponding histology with H&E staining; (c-f) representative TEMs of cells from the basal layer (c), prickly layer (d), intermediate layer (e) and keratinized layer (zoomed-in view of rectangular area in f) at 50,000 $\times$ magnification, respectively. Green and orange arrows indicate keratin filament bundles and individual keratin filament, respectively; red arrows indicate cell borders. N: nucleus. Scale bars for μ OCT and H&E stained images, 50 μ m; scale bars for TEMs, 0.5 μ m.

Note that the basal layer of nonpigmented SSEs sometimes seemed to be low scattering

although filaments in basal cells were also packed into electron dense bundles (**Figure 5. 8c, Figure 5. 9c & Figure 5. 10c**). It is possibly owing to the fact that these cells have large nucleocytoplasmic ratio and are generally crowded to each other so that the scattering feature at this region is dominated by that of nuclei. Besides, imaging artifacts such as light attenuation may also contribute to its low optical intensity relative to the overlying epithelial layers. Also, the keratinized layer in orthokeratinized SSE could demonstrate either high intensity or low intensity (**Figure 5. 8a**). The low intensity was possibly due to the fact that these terminally keratinized cells were highly dehydrated, compact, and filled only with keratin filaments, rendering a homogenous distribution of refractive index (**Figure 5. 8f**).

5.3.3.2 Reflectance contrast abnormalities in the orthokeratinized mouse esophagus with intraepithelial neoplasia

μ OCT images acquired from hyperplastic epithelium resembled the features observed in the normal orthokeratinized epithelium: well differentiated keratinocytes demonstrated high-intensity cytoplasm and detectable nucleocytoplasmic contrast covered by a well-developed keratinized layer (**Figure 5. 11 a1&a2**). However, images obtained from regions with pathologically confirmed severe dysplasia showed a relatively low optical intensity occupied by immature dysplastic cells with enlarged nuclei and little cytoplasm (**Figure 5. 11 b1&b2**, white stars), in contrast to the neighboring region with mild dysplasia where cells particularly in the upper two-thirds of the epithelium were well differentiated with rich cytoplasm (**Fig 11 b1&b2**, white arrows).

5.3.3.3 Reflectance contrast abnormalities in the nonkeratinized human esophagus with intraepithelial neoplasia

In the μ OCT images acquired from the Lugol's-positive clean margin of human esophageal specimen, we could read out characteristic architectural and cytologic information of the normal nonkeratinized epithelium (**Figure 5. 11 c1&c2**). Keratinocytes at the intermediate layer presented relatively low-intensity cytoplasm similar to those observed in nonkeratinized floor of mouth (**Figure 5. 11c1**). By contrast, in the images obtained from Lugol's-negative area that was confirmed to be severe dysplasia pathologically, the optical intensity differences divided the epithelium into 2

portions: the surface high-intensity portion and the underlying low-intensity portion (**Figure 5. 11 d1,d2**). According to the corresponding histology (**Figure 5. 11 d2**), the high-intensity portion was consisted of cells with flatted nuclei and rich cytoplasm indicating well cell differentiation and the underlying portion was occupied by cells with oval-shaped enlarged nuclei suggesting impaired cell maturation.

Figure 5. 11 Subcellular reflectance contrast of the mouse (a1-b2) and human (c1-d2) esophagus with intraepithelial neoplasia imaged by μ OCT *ex vivo*. (a1, b1, c1, and d1) Representative μ OCT cross-sectional images and (a2, b2, c2, and d2) matched histology with H&E staining: (a1, a2) a mouse specimen

with hyperplasia; (b1, b2) another mouse specimen with intraepithelial neoplasia where white arrows indicate mild dysplasia and white stars indicate severe dysplasia; (c1-d2) a patient specimen confirmed with severe dysplasia acquired from the clean margin (c1, c2) and the neoplastic region (d1, d2): Red arrowheads indicate boundary between epithelium and lamina propria and red arrows suggest low-scattering nuclei. Scale bars, 50 μm .

5.4 Discussion and Conclusion

OCT technology is rapidly advancing towards optimal clinical utilities from bulky bench-top workstation to compact probe- or capsule-based endomicroscopy, which will open up new avenues for the screening and detection of epithelial disorders^{13-15,65-68}. Improving our understandings of the information encoded in the images is one of the fundamental steps to realize the potentials of this promising technology. The present study uncovers that nanometer-scale organelles such as mucin granules, keratin filaments and microvilli are significant or main sources of back-scattered signals, which has never been reported before. The FDTD simulation results reveal the correlations between the back-scattering contrasts and the ultrastructural clustering or packing states of the organelles, which are validated by the measured data in μOCT images. All these results establish that the reflectance contrasts of μOCT images could be used to decode the physiological/pathophysiological states of epithelial cells, a new step forward towards the long sought goal of ‘virtual histology’.

Our results for the first time open up the possibility to distinguish different mucin-secreting cells that are similar in morphology and function in intact tissues without the need of exogenous contrast agents, which has not been proven possible despite the resolving power has been pushed to the cellular level^{35,69}. The study indicates that this advance could reasonably help with the detection of intestinal metaplasia (IM) in Barrett’s esophagus and stomach^{70,71}, which is challenging to be targeted with traditional video-imaging modalities even when a rigorous “systematic biopsy” protocol is used due to inadequate contrast between IM and surrounding epithelium^{2,17}. Besides, as cellular information could be differentiated based on intrinsic contrast, it would possibly eliminate labor-intensive staining procedures and concerns on the cytotoxicity of contrast agents^{69,72}. In addition, although human foveolar cells contain exclusively of

neutral mucins which is different from those of swine, their apical portion also present high optical intensity. This agreement in the back-scattering features of foveolar cells is possibly owing to that they share a similar ultrastructural feature in the mucin granules⁷³.

The characterization of colon polyps exemplifies another clinical benefit to understand the subcellular reflectance contrast. With the expanding implementation of colonoscopy for cancer screening, colorectal polyps are increasingly detected⁷. However, since more than 90% polyps are small with approximately 50% of them being non-neoplastic^{7,74,75}, pathologic assessment is in most cases used only to determine surveillance intervals^{7,75}, which gives rise to significant healthcare costs^{7,76}. Based on the understanding of reflectance contrasts established in this study, we show that pathology hallmarks like decreased goblet cells, pseudostratified nuclei and irregular crypt patterns in adenomatous polyps can be well recognized in μ OCT images. This capability may offer a possibility to differentiate adenomatous polyps from non-neoplastic polys in real time, which would possibly save substantial pathology costs in colon cancer prevention^{7,76}.

The current study also provides us a comprehensive understanding of the reflectance signals from nonpigmented keratinocytes. We disclosed that the cytoplasmic reflectance intensity was correlated with the packing state of keratin filaments. With the biological bases underlying subcellular reflectance contrast of keratinocytes clarified, we demonstrate the capability to distinguish between differentiated cells and dysplastic cells in both the orthokeratinized and nonkeratinized SSEs. Since the packing state of keratin filament is associated with the level of keratinization, this knowledge may also be useful for grading cancer cells in the squamous cell carcinoma because the synthesis as well as the packing state of keratin filaments varied between well-differentiated and poorly-differentiated neoplastic cells⁷⁷. Additionally, the reflectance contrast difference between the squamous and columnar epithelium at the squamocolumnar junction suggests a possibility to detect squamous metaplasia in the simple columnar epithelium, which is a pre-neoplastic change in organs like cervix and lung⁷⁸.

While few tools are available in optimally evaluating brush border except for histology, we demonstrate the possibility to evaluate this polarization-derived subcellular structure using μ OCT noninvasively and in real-time. This ability may benefit the research on a number of microvilli related abnormalities, such as celiac disease⁷⁹ and microvillus inclusion disease⁸⁰.

Back-scattered contrast depends on complex processes of Mie and Rayleigh scattering, involving relative refractive indices, shape and orientation and clustering of structures, etc. The contrast seen in optical reflectance images is due to an ensemble of scattering events – those in the optical section that are modulated by those in the tissue layers above and below. Our numerical models were developed based on multiple assumptions in the shape, orientation, distribution, and refractive index of the clustered and packed scatterers. These simplification strategies are necessary since some of these data is not available or measurable. In our study, this shortcoming is compensated by the experimental validations. However, our numerical modelling cannot include all the biological variations of the scatterers under investigation, such as keratin filaments in the keratinized layers and basal layers of the stratified squamous epithelium. In these cases, speculative observations are reported based on our experiences.

Our preliminary study provided promising results on distinguishing precursor lesions from normal mucosa, but future multicenter studies with large sample size from various tissue types would be necessary to validate the efficiency and accuracy of these criteria. In addition, it is worth mentioning that correlation does not imply causation since biology is rather complex and there are still a lot of unknown need to be disclosed. It is also worth mentioning that although TEM images serve as the “ground truth” of the accumulation and distribution of intracellular components, TEM contrast may not directly correlate with reflectance contrast since TEM contrast is offered by the affinity between tissue components and contrast agents (uranyl acetate and lead citrate), whereas OCT contrast is provided by refractive-index heterogeneities.

In conclusion, this part of work elucidates the ultrastructural bases underlying optical reflectance (back-scattering) contrast using both numerical and experimental approach. Although demonstrated with μ OCT, these understandings on the biological meaning of reflectance contrast should be directly applicable to other optical reflectance techniques including but not limited to RCM^{41,72}, OCM³⁵ and FF-OCT³⁷. Looking forward, with the rapid advances in the development of cellular-resolution optical reflectance technologies^{15,16,81,82}, diagnostic criteria based on these new understandings would complement the current morphology- or architecture-based criteria to specifically decode the pathophysiological events within tissues. Combined with effective algorithm such as machine learning method, it could be possible to characterize these cellular

components automatically. The success in these efforts would ultimately have a substantial impact for the screening and surveillance of epithelial diseases by reducing costs and risks from unnecessary resections and sampling errors.

Chapter 6

Subcellular Structures in Cornea with μ OCT Imaging

6.1 Introduction and Background

Cornea serving as the physical barrier of the eye is a transparent, layered-structure tissue (**Figure 6.1 A schematic of corneal structure** (reproduced from reference⁸⁸)). Corneal homeostasis can be perturbed by a variety of pathological conditions, such as trauma, infection and endothelial dystrophy, resulting in corneal edema or haze^{9,83}. Second only to cataract, corneal disease is a major cause of visual impairment worldwide^{8,9} and corneal transplantation remains the most effective method for visual restoration⁹. In particular, up to 50% of all corneal transplantations are performed to treat endothelial dystrophy, a disorder that destroys corneal epithelial cells irreversibly^{84,85}. Recent understanding of corneal anatomy has contributed to an innovation in corneal transplantation in which the pre-Descemet's layer (PDL) together with Descemet's membrane (DM) and endothelium are transplanted, decreasing technical complexities in endothelial keratoplasty and avoiding postoperative complications in penetrating keratoplasty^{10,11}. Consequently, accurate delineation of corneal layers and the corneal allograft interface would be of great significance in pre-, intra-, and post- operative evaluation. On the other hand, the quality of donor endothelial cells is the critical parameter that determines corneal graft survival in corneal transplantation¹², and it remains a challenge for eye banks to optimize storage strategies to maximally preserve viable endothelial cells and other corneal components, especially in areas that face a shortage of donor corneas^{86,87}. Successful and efficient visualization of cellular and extracellular components *in situ* would provide valuable information for longitudinal assessment of eye bank corneas.

Figure 6. 1 A schematic of corneal structure (reproduced from reference⁸⁸).

Noncontact specular microscopy (SM) is most commonly used for the assessment of endothelial cells; however, information is limited to the posterior surface of the endothelium and even mild corneal edema may lead to blurred images^{89,90}. *In vivo* confocal microscopy (IVCM) allows visualizing corneal layers at the cellular level and is not sensitive to slight corneal edema^{91,92}. Whereas, it is difficult to differentiate those thin layers at the posterior cornea owing to limited axial resolution ($4\text{--}25\text{ }\mu\text{m}$)⁹². In addition, the high transverse resolution ($< 1\text{ }\mu\text{m}$) is achieved at the expense of field of view ($\sim 400\text{ }\mu\text{m} \times 400\text{ }\mu\text{m}$), thus making it troublesome for repeated investigation of the same area over time^{93,94}. Full-field optical coherence tomography (FF-OCT), which allows for both transverse and axial resolution at the cellular level ($\sim 1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m}$), has emerged as an alternative to IVCM⁹⁵⁻⁹⁷. However, its long image acquisition time ($\sim 1\text{--}1.5\text{ s}$ per *en face* frame) makes it unsuitable for *in vivo* corneal imaging^{85,96}. Optical coherence tomography (OCT) is a powerful tool for evaluating corneal structures because its axial resolution is decoupled from its transverse resolution and is determined by the center wavelength and bandwidth of the light source¹³. However, the axial resolution of commercially available anterior segment OCT (AS-OCT) and custom-built ultrahigh resolution OCT (UHR-OCT) instruments is limited to $5\text{--}20\text{ }\mu\text{m}$ and $3\text{--}5\text{ }\mu\text{m}$, respectively, which is still insufficient to differentiate the DM and endothelial layer⁹⁸⁻¹⁰⁰. Bizheva *et al.* have recently used an UHR-OCT with a resolution of $1 \times 5\text{ }\mu\text{m}$ (axial $\times$ lateral resolution) to successfully delineate the PDL, DM and endothelial layer¹⁰¹. However, none of these OCT systems have sufficient lateral resolution to resolve cellular level microstructures from an *en face* plane.

Micro-OCT (μOCT), with an isotropic spatial resolution of $1\text{--}2\text{ }\mu\text{m}$, can detect key cellular and subcellular components associated with arteriosclerosis, pulmonary airway disease, and intracochlear defects *ex vivo*^{19,102,103}. More recently, we have tested its

performance in imaging hexagonal corneal endothelial cells by using a rat model⁸⁵. However, the ability of μ OCT to enable visualization of micro-anatomical structures of the posterior cornea has not been fully explored. We here present micro-anatomical structures of the posterior cornea in mice, rats and swine captured by μ OCT *ex vivo* and *in vivo*. Some of these structural features, to the best of our knowledge, are reported for the first time, which should contribute to understanding of the corneal anatomy associated with corneal transplantation.

6.2 Materials and Methods

6.2.1 Tissue Preparation and Image Acquisition

We conducted μ OCT imaging of corneal tissues in mice, rats and swine. Two C57/bl6 mice (female, 8-9 weeks old) and 4 Sprague Dawley rats (female, 12-14 weeks old) were used for *ex vivo* study, and 2 Sus Scrofa minipigs (female, 2-3 years old) were used for *in vivo* and *ex vivo* study. For *ex vivo* studies, eyes of laboratory animals were immediately enucleated after sacrifice and washed using buffered saline (PBS) (pH=7.4, Gibco[®] by Life Technologies). Subsequently, each enucleated eye was placed into a custom-designed container with the corneal side up and transferred onto the scanning stage of the μ OCT system for image acquisition. All images of the central cornea were captured by one researcher. After image acquisition, eyes were fixed with neutral-buffered formalin (4% formaldehyde; Leica Biosystems Richmond Inc.) for histological analysis. In addition, to test the capability of μ OCT to longitudinally monitor the status of endothelial cells in corneal grafts, enucleated rat eyes were stored in PBS at 4°C, and image acquisition was repeated at <5 mins and at 2, 4, 8, and 24 hours after harvest. For *in vivo* studies, experimental animals were mounted on the scanning stage for image acquisition while they were under general isoflurane anaesthesia. The study was approved by the Institutional Animal Care and Use Committee of Nanyang Technological University, Singapore (ARF-SBS/NIE-A0312) and the Institutional Animal Care and Use Committee of PWG Genetics Pte Ltd., Singapore (PN16076). All included animals were treated according to the statement of the Association for Research in Vision and Ophthalmology regarding the Use of Animals in Ophthalmic and Visual Research.

6.2.2 Data Acquisition and Statistical Analysis

Endothelial cell density of normal mice, rat and swine was evaluated using fixed-frame analysis⁹⁰. Average endothelial thickness and DM thickness of each species were manually quantified based on 100 randomly selected positions of each three dimensional dataset. To compare CCT at different time points after harvest (<5mins, 2, 4, 8 and 24 hours), 32 images were selected from 1024 images with an interval of 32 b-scan frames for quantitative analysis. One-way analysis of variance (ANOVA) was used to compare corneal thickness among different groups, and Bonferroni post hoc tests were performed for multiple comparisons when statistical significance was recognized. All numerical values are presented as the mean $\pm$ standard deviation (SD), and $p < 0.05$ was considered to indicate a statistically significant difference. All statistical analysis was performed using SPSS software (IBM SPSS Statistics 23.0).

6.3 Experimental results

6.3.1 Mice cornea

Representative *ex vivo* cross-sectional μ OCT images of mice corneas showed well-defined interfaces between adjacent posterior corneal layers (**Figure 6. 2a**), as confirmed by corresponding histological images (**Figure 6. 2c**). The interface between the aqueous humour and endothelium, corresponding to the apical side of endothelial cells, resulted in a continuous hyper-reflective line. The interface between the endothelium and DM, corresponding to the basolateral side of endothelial cells, appeared as an interspaced high scattering string. Each high scattering segment of the string appeared to be dome-shaped (red arrow in **Figure 6. 2a**) and corresponded to one endothelial cell (**Figure 6. 2b**). The region sandwiched by the bright apical and basolateral borders which should correspond to the cell body was relatively low-scattering. DM was hypo-reflective in μ OCT images with a high scattering interface with the stroma (**Figure 6. 2a**). The tomographic view of the stroma was optically heterogeneous with highly scattering linear structures, running parallel to the surface of the cornea against a low reflective background. We speculated that these linear structures were collagen bundles detectable in the near infrared spectrum by μ OCT. The average thickness of endothelium and DM was $5.31 \pm 0.58 \mu\text{m}$ and $1.96 \pm 0.59 \mu\text{m}$, respectively.

Polygonal or hexagonal endothelial cells were clearly delineated with distinct low reflective cell boundaries with cell density of 952 per mm² (**Figure 6. 2d&g**). The dark spots located at the apical surfaces of endothelial cells were believed to be the root of primary cilia rather than cell nuclei, as previously speculated (red arrows in **Figure 6. 2g**)¹⁰⁴. This hypothesis was supported first by the observation that they were too small for nuclei^{85,104} and second by the observation that the dark spots were found at only the apical side of the cell membrane, not at both the apical and basolateral sides. Interestingly, the *en face* images of the basolateral surface of endothelial cells demonstrated a hyper-reflective lattice, a finding that has never been reported on the basis of confocal microscopy or FF-OCT data (**Figure 6. 2e&h**). Orthogonal projections of this hyper-reflective lattice showed that each junction point of the lattice corresponded to one dome-shaped endothelial cell. This observation was confirmed when we overlaid the image of the endothelial apical surface with the lattice: a one-to-one match was observed between each intersection point and endothelial cell centre on the overlapped image (**Figure 6. 2j-l**). The posterior stroma seen from the *en face* view consisted of hyper-reflective stellate keratocytes along with linear structures against a hypo-reflective background (**Figure 6. 2f&i**). Analogous to the observations with confocal microscopy and electron microscopy, keratocytes in the μ OCT images were stellate with one large body and several ramified processes (red asterisks in **Figure 6. 2f&i**)^{105,106}. Those linear highly reflective structures was suggestive of cell processes of keratocytes, collagen fibres or nerve fibres (yellow arrows in **Figure 6. 2f&i**).

Figure 6. 2 Ex vivo μ OCT imaging of the mouse cornea. (a) Cross-sectional μ OCT image of mouse cornea. Inset is the zoomed-in view of the rectangular area; red arrows indicate endothelial cells. (b) Scheme of the tomographic view of endothelial cells. (c) Cross-sectional histological image of mouse cornea. (d) *En face* view of the apical side of the endothelium demonstrated regularly arranged polygonal cells with low reflective cell boundaries. (e) *En face* view of the interface between the endothelium and DM, corresponding to the basolateral side of the endothelium, presented a high scattering lattice. (f) *En face* view of posterior stroma. Stellate keratocytes (red asterisks) and linear collagen fibres

(yellow arrows) were both highly reflective. **(g-i)** Zoomed-in view of the square region in **(d-f)**. Dark spots are probably primary cilia of endothelial cells (red arrows in **g**). **(j)** Apical surface of endothelial cells. **(k)** Basolateral surface of endothelial cells. **(l)** Overlap of apical and basolateral surface of endothelial cells. Ep: epithelium; BL: Bowman's layer; S: stroma; DM: Descemet's membrane; Ed: endothelium; EDC: endothelial cell (Scale bar=50 μm and scale bar of inset in **(a)** represents 25 μm).

6.3.2 Rat cornea

Similar to those observed in mice, corneal layers including endothelial layer, DM layer could be readily recognized in the cross-sectional μOCT images *ex vivo*, matched well with corresponding histology (**Figure 6. 3a&b**).

Figure 6. 3 *Ex vivo* μOCT imaging of the rat cornea. **(a)** Cross-sectional μOCT image of rat cornea. Inset is the zoomed-in view of the rectangular area; red arrows indicate endothelial cells. **(b)** Cross-sectional histological image of rat

cornea. (c) *En face* view of the apical side of the endothelium demonstrated regularly arranged polygonal cells with low reflective cell boundaries. (d) *En face* view of the interface between the endothelium and DM, which corresponded to the basolateral side of the endothelium, presented a highly scattering lattice. (e) *En face* view of posterior stroma. Stellate keratocytes (red asterisks) and linear collagen fibres (yellow arrows) were both visualized. (f-h) Zoomed-in view of the square region in (c-e). Dark spots are probably cilia of endothelial cells (red arrow in f). Ep: epithelium; BL: Bowman's layer; S: stroma; DM: Descemet's membrane; Ed: endothelium (Scale bar = 50 μm and scale bar of inset in (a) represents 25 μm).

The average thicknesses of endothelium and DM were measured to be $4.78 \pm 0.43 \mu\text{m}$ and $3.23 \pm 0.46 \mu\text{m}$, respectively. Continuous *en face* views of posterior cornea from 3D reconstruction of *ex vivo* μOCT data are presented in **Figure 6. 3c-h**. Polygonal or hexagonal endothelial cells with detectable dark spots at the apical surface (**Figure 6. 3c&f**) and a hyper-reflective lattice at the basolateral surface of the cells could be recognized on the μOCT images (**Figure 6. 3d&g**). Endothelial cell density was 1108 per mm^2 . Hyper-reflective stellate keratocytes (red asterisks in **Figure 6. 3e&h**) and linear structures (yellow arrows in **Figure 6. 3e&h**) against a hypo-reflective background could also be identified on the *en face* view of μOCT images.

6.3.3 Swine cornea

μOCT cross-sectional images of swine acquired *in vivo* clearly delineated the posterior corneal layers, matched well with corresponding histology (**Figure 6. 4a&b**). In addition, we also observed a thin high scattering layer just anterior to the DM in swine, which was reminiscent of the pre-DM layer (PDL) (**Figure 6. 4a**). The endothelial cells with dome-shaped basolateral surface could still be identified on the cross-sectional μOCT images (red arrow in **Figure 6. 4a**). The average thicknesses of endothelium and DM were $4.78 \pm 0.70 \mu\text{m}$ and $32.42 \pm 1.11 \mu\text{m}$, respectively. On the *en face* μOCT images *ex vivo*, the polygonal endothelial cells could be recognized with a measured density of 2139 per mm^2 (c). Comparable to second-harmonic generated (SHG) images¹⁰⁷, collagen fibres in swine corneal stroma tended to extend in parallel collagen bundles, interweaving with one another at specific orientations, and the density of

visible collagen bundles gradually increased as they reached the DM (yellow arrows in **Figure 6. 4d-f**). Occasionally, we could identify high scattering structures forming “Y”-shape or asterisk-shape which were probably nerve fibres (yellow triangle in **Figure 6. 4f&g**).

Figure 6. 4 μOCT imaging of the pig cornea. (a) *In vivo* cross-sectional μOCT image of swine posterior cornea. Inset is the zoomed-in view of the rectangular

area; red arrows indicate endothelial cells. Corneal layers were clearly delineated, and collagen bundles in stroma were also visualized. **(b)** Cross-sectional histological image of posterior cornea. **(c)** *Ex vivo en face* view of the apical side of the endothelium presented regularly arranged polygonal cells. **(d-f)** Representative *ex vivo en face* views of posterior stroma from the lower to upper level. Both keratocytes (red asterisks) and collagen bundles (yellow arrows) were visualized, and the density of visible collagen bundles decreased from the most posterior to upper posterior stroma. Yellow triangles in **f** and **g** indicate nerve fibres in different patterns. S: stroma; PDL: pre-Descemet's layer; DM: Descemet's membrane; Ed: endothelium (Scale bar=50 μm and scale bar of inset in **(a)** is 25 μm).

6.3.4 Corneal subcellular structures degeneration

We also longitudinally observed the scattering variations of posterior cornea to determine whether μOCT is effective for monitoring endothelial cell status after enucleation and μOCT images of the central cornea of the same rat were acquired at <5 mins and at 2, 4, 8, and 24 hours after harvest. Similarly to images acquired within 5 mins (**Figure 6. 5 a1,b1&c1**), the *en face* view of the posterior cornea captured at 2 hours demonstrated regularly arranged polygonal endothelial cells with dark spots at the apical surface, a lattice structure at the basolateral surface, and high scattering collagens and keratocytes in the stroma (**Figure 6. 5 a2,b2&c2**).

After 4 hours, the area of the low-scattering (dark) spots significantly increased, presenting an early sign of endothelial defect (rectangular area in **Figure 6. 5 a3**). The lattices at the basolateral surfaces of endothelial cells as well as collagen fibres and keratocytes in the posterior stroma were all well preserved (**Figure 6. 5 a3,b3&c3**).

After 8 hours, the plane of the endothelial apical surface was no longer as flat as it was at <5 mins. Several pimple-like or dome-shaped endothelial cells scattered amongst the flat hexagonal endothelial cells, indicating swelling of endothelial cell which provided evidence of endothelial decompensation (yellow arrows in **Figure 6. 5 a4 & Figure 6. 6a**). The rest corneal components, including collagen and keratocytes, in the posterior cornea were well preserved (**Figure 6. 5 a4,b4&c4**).

Figure 6. 5 Longitudinal *ex vivo* observation of the rat central cornea with μ OCT. *En face* views taken at <5 mins (a1-c1), 2 h (a2-c2), 4 h (a3-c3), 8 h (a4-c4) and 24 h (a5-c5). (a1-a5) *En face* view of apical side of endothelial cells. Green square insets are zoomed-in view of red square areas. Yellow arrows in

a4 and **a5** indicate endothelial cell swelling, which changed the cell surface from flat to dome-shaped. (**b1-b5**) *En face* view of the basolateral surface of endothelial cells and the interface between endothelium and Descemet's membrane. A high scattering lattice was detectable at all these time points. (**c1-c5**) *En face* view of posterior stroma. Highly reflective collagen fibres and keratocytes are both visible at different time points, whereas the density of collagen fibres appears to decrease at 24 hours after sacrificed. (Scale bar=50 μ m).

After 24 hours, it was difficult to differentiate cell boundaries owing to the clustered swelling of endothelial cells (rectangle area and yellow arrows in **Figure 6. 5a5**). In addition, the density of collagen fibres was clearly decreased, indicating that pathological changes occurred in the stroma (**Figure 6. 5c5**). Clearly presented in the cross-sectional μ OCT images, endothelial cell swelling (**Figure 6. 6a**) and cell defects appearing as a low-scattering gap at the apical surface could be readily recognized (**Figure 6. 6b**). Several leukocytes in the anterior chamber were adhered to the endothelium, and the attachment point was located right at the cell boundary (red arrow, **Figure 6. 6c**). This phenomenon indicated that leukocytes infiltrated the cornea via the interface between endothelial cells.

Figure 6. 6 Three-dimensional view of deformation of endothelial cells. (a) Red arrow indicates dome-shaped deformation of endothelial cells at the apical surface. (**e**) 3D view of endothelial defect (red arrows). (**f**) 3D view of leukocyte infiltration into the cornea from cell boundaries (red arrow) (Scale bar=50 μ m).

In addition to morphological observation, total central corneal thickness (CCT), which is an important parameter in evaluation of endothelial function, was manually measured. The CCTs of rat cornea at <5 mins and at 2, 4, 8 and 24 hours were $189.12 \pm 1.83 \mu$ m,

223.41 $\pm$ 0.84 μm , 250.01 $\pm$ 1.01 μm , 281.22 $\pm$ 1.34 μm and 354.72 $\pm$ 1.08 μm , respectively. One-way ANOVA and post hoc multiple comparisons indicated a statistically significant increase in CCT as early as 2 hours after enucleation ($p < 0.001$).

6.4 Discussion and Conclusion

This study demonstrates the scattering features of cellular and extracellular components in the posterior cornea and clarifies what information μOCT can provide in corneal imaging, a question that has not been fully answered by previous studies. Second, but more importantly, we observe the micro-anatomic characteristics of the posterior cornea that are shared among all three species *in situ* which have not previously been reported. On the basis of the existing and new micro-anatomical information uncovered by μOCT , this study further explore the clinical utility of μOCT in the context of corneal transplantation. The ability of μOCT to provide both high isotropic spatial resolutions and high imaging speed makes it a unique tool for this task. Last, we conducted *in vivo* μOCT imaging in swine corneas to confirm our *ex vivo* findings.

Similar to those observed by 0.95- μm -axial-resolution OCT developed by Bizheva,¹⁰¹ μOCT located posterior corneal layers including the PDL (visible in swine cornea), DM and endothelium accurately which allows for precise separation of corneal layers and has valuable significance in corneal graft preparation and postoperative assessment^{9,101}. Moreover, precise delineation of corneal layers will also contribute to the long-term study of the natural history of endothelial decompensation in diseases such as Fuch's endothelial dystrophy^{11,84}. More interestingly, the μOCT cross-sectional images clearly show that endothelial cells have a dome-shaped basolateral side facing the DM and a flat apical side facing the aqueous humour, analogous to observations in *in vitro* cultured endothelial cells¹⁰⁸, where the dome-shaped pattern may be partly owing to the endothelial nucleus¹⁰. Although the shape of corneal endothelial cells has been thoroughly investigated by histology, this is the first time that the detailed morphology of endothelial cells is uncovered in intact corneal tissue under their natural conditions both *ex vivo* and *in vivo*. This new natural corneal morphological feature may open up a new approach to efficiently evaluate endothelial cell status.

In addition to polygonal endothelial cells at the apical surface, a lattice was observed at

the basolateral side of the endothelial cells, which has not previously been reported with confocal imaging or FF-OCT^{94,96,97,106}. The inability to detect such a lattice with IVCN is probably due to its insufficient axial resolving power^{94,106}. Although it has not previously been described, the network-like structure which enriches our knowledge of corneal anatomy deserves further research to reveal its physiological function and clinical meaning. The cellular-level *en face* images together with the cross-sectional view demonstrate μ OCT's possibility to provide morphological endothelial cell information in three dimensions. Thus, it may become an alternative for evaluation of eye bank corneas, especially for the assessment of endothelial cells, which is currently performed by conventional light microscopy, SM and IVCN^{12,109}. In this study, we also performed a preliminary study with rat eyes to determine whether μ OCT could be used to monitor pathological processes in enucleated corneas. Images captured at different time points indicate that μ OCT is able to detect several signs of endothelial cell degeneration at an early stage in three dimensions. This capability would directly aid in pre- and postoperative evaluation of corneal grafts used for corneal transplantation.

In addition to comprehensive interpretation of endothelial cells, μ OCT also has the ability to detect cellular components in the stroma. In this study, we focused on the posterior stroma which is anatomically close to the endothelium and DM. Similarly to IVCN^{94,106}, μ OCT also can be used to visualize keratocytes, and the visibility of keratocytes allows for cell density assessment, which may accordingly aid in diagnosis of diseases such as keratoconus. μ OCT is superior to IVCN because it also has the capacity to detect collagen fibres in stroma, and the visibility of collagen may be attributed to the difference in refractive index between collagen ($n=1.41$) and the corneal extracellular matrix ($n=1.35$)¹¹⁰. Observation of collagen fibres is helpful for the understanding of the relationship between collagen orientation and corneal shape. Moreover, it may also aid in longitudinal analysis of diseases such as corneal ectasia, which presents as disorganization of collagen fibres in stroma¹¹¹.

The limitations of current μ OCT technology for imaging corneas *in vivo* include short depth of focus of the system and motion artefacts in the reconstructed *en face* images. To achieve cellular level transverse resolution, the depth of focus (DOF) of the current μ OCT systems is ~ 30 - $40\ \mu\text{m}$, thus making it difficult to keep the area of interest within the depth of focus, owing to the axial motion of eyes. The DOF issue may be resolved

by depth of focus extension techniques^{68,112-114}. However, it's worth mentioning that the axial field of view, defined as the axial range of 6 dB intensity roll-off is much larger than the DOF (~115 μm and 32.6 μm in air respectively). As a result, in log scale images, landmark features of corneas can still be clearly visualized over the entire cornea though at a lower lateral resolution. In addition, insufficient image acquisition speed with an A-line rate at 60 kHz (60 frames/s) remains a barrier for *in vivo en face* image analysis. The quality of *en face* images of corneal endothelium reconstructed from the 3D dataset was significantly compromised by the eye motion, and thus, neither apical or basolateral cellular structures could be clearly identified. An effective motion tracking algorithm (such as deep learning algorithm) and improvement in μOCT image acquisition speed may be promising solutions for decreasing motion artifacts¹¹⁵.

In summary, all these findings of the cellular and subcellular components of posterior cornea provide new opportunities for improving our understanding of corneal anatomy. Looking forward, with the modification of system performances in parameters such as ranging depth (eg. a IIS design) and imaging speed, non-invasive μOCT would be a powerful tool for tracing the natural history of disease development and helping with corneal disease diagnosis and treatment such as in corneal transplantation.

Chapter 7

Conclusion and Recommendations

7.1 Conclusion

We in this thesis recognize the potentials of OCT in translation to epithelial disease screening by improving our understandings on the histopathological significances encoded in the OCT back-scattered contrasts. A fiber-based cellular-resolution SD-OCT (μ OCT) system with a handheld probe is developed, suitable for the image acquisition of epithelial tissues both *ex vivo* and *in vivo*. Using this μ OCT system, the experimental evidences on the subcellular scattering features of nuclei and cytoplasm are evaluated and validated in the full spectrum of epithelial tissues including both scattering and transparent tissues from small and large animals.

We clarify that the nuclei of epithelial cells present low-scattering signals and those previous reported ‘bright’ nuclei against a ‘dark’ cytoplasmic background are actually the nucleocytoplasmic boundaries with μ OCT operating at 800 nm. It is the insufficient axial resolution of the imaging systems that limits the capability to resolve the dark nuclear core. This result unifies the inconsistent reports on the scattering characteristics of nuclei and more importantly will contribute to the consensus in the establishment of OCT diagnostic criteria. We also disclose that the scattering features of the cytoplasm is cell-type specific. Our results indicate that differently differentiated or specialized epithelial cells could demonstrate varied cytoplasmic scattering and nucleocytoplasmic contrast. All those new understandings on optical reflectance signals will help with decoding the pathophysiological events underneath epithelial surfaces by recognizing histology-matched information in real-time, which will make it possible for clinicians to make a diagnosis during on-going examination. In transparent corneal tissues, the scattering features of the monolayer epithelium (i.e. endothelium) as well as the corneal subcellular structures disclosed with μ OCT offer us the opportunities to trace the natural

histology of endothelium-related diseases, and aid in pre- and post-operative evaluation of corneal status and graft survival in corneal transplantation.

In conclusion, with the accumulating achievements in cellular-resolution optical reflectance imaging technology including but not limited to OCT, confocal reflectance microscopy and optical coherence microscopy, the clarifications on the reflectance contrast of cells will allow immediate characterization of epithelial abnormalities, which may have an important significance for the screening and surveillance of epithelial diseases including cancers.

7.2 Recommendations for Further Research

7.2.1 Establishment and Validation of OCT Diagnostic Criteria

With the decoding of the histopathological significances underlying the subcellular reflectance contrasts, further studies are required to establish cellular-resolution OCT diagnostic criteria and to validate its sensitivity and specificity in identifying epithelial diseases, in particular, precancerous lesions in clinical suites.

A case-control clinical study will be designed to establish the OCT diagnostic criteria for intestinal metaplasia (IM) in stomach, which is a common precancerous lesion. Patients underwent endoscopic resection of gastric suspicious lesions in the epithelium at the department of gastroenterology in a hospital will be enrolled in this study. Fresh specimens will be sent for OCT imaging immediately after the resection prior to histopathological procedure. For each specimen, OCT imaging will be conducted firstly with the low-resolution but wide-field scanning mode and then focus on the suspicious regions with the cellular-resolution mode. Each suspicious lesion will be marked with tissue-marking dye with different colors if multiple lesions are detected in one specimen.

The diagnostic criteria of gastric IM is expected to be established by imaging the first 20 specimens which are confirmed with IM histopathologically. The sensitivity and specificity of the OCT criteria in charactering gastric IM will be evaluated with histology being the gold standard as shown in **Table 7. 1**.

Table 7. 1 Sensitivity and Specificity of OCT criteria in characterizing gastric

intestinal metaplasia*

OCT	Histology (gold standard)		Total
	Positive	Negative	
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	a+b+c+d

*sensitivity = $a/(a+c)$; specificity = $d/(b+d)$

7.2.2 Spectrometer Design with Improved Axial Range and Reduced Cost

Although our self-designed μ OCT system demonstrates satisfactory performances in resolving subsurface microstructures with $<2 \mu\text{m}$ spatial resolutions, there are considerable rooms for improvement. For example, the ranging depth is so limited that it is troublesome to acquire a high-quality 3D OCT dataset particular when the tissue surface is not flat. In the future, a spectrometer with higher ranging depth ideal at a low cost will be designed.

Instead of modifying the μ OCT, we use the ultrahigh-resolution OCT system with conventional spectrometer which has been stated in Chapter 3.2 as the benchmark. In that system, the spectrometer consists of a collimation lens L5 (AC127-030-B, Thorlabs Inc., USA), a grating (1200 l/mm, WP-1200/840-50.8, Wasatch Photonics Inc., USA) with a center-wavelength diffraction efficiency of $\sim 86\%$, a camera lens (Nikon AF Nikkor 85 mm f/1.8D), and a line scan camera (CCD, E2V, EV71YEM4CL2014-BA9) with a pixel size of $14 \mu\text{m} \times 28 \mu\text{m}$ (width $\times$ height) and a pixel number of 2048.

Figure 7. 1 Design of a high-efficiency with low-cost spectrometer.

In this new spectrometer design as shown in **Figure 7. 1**, the expensive Nikon camera lens will be replaced with two spherical singlets (plano-convex lenses), which is much cheaper (<\$50) and, more importantly, reduced the measured signal loss from ~20% to <1.5%. We also replace the doublet collimation lens with an off-axis parabolic mirror. The grating will be replaced with a higher-efficiency one (diffraction efficiency >92%; 1500 l/mm, Ibsen Photonics). Based on this spectrometer design, the 6-dB roll-off range of the input light is measured to be 50% larger than that provided by the conventional spectrometer which is clearly presented in **Figure 7. 2**. In the future, the performance of this new design of spectrometer will be tested in μ OCT system.

Figure 7. 2 The 6-dB roll-off range comparison between the old (a) and newly-designed (b) spectrometer.

Moving forward, we will continue to optimize the optical and mechanical design in the following aspects: 1. Develop a linear- k (wavenumber) spectrometer by adding a prism to correct the k -space nonlinearity problem caused by the grating; 2. Design and fabricate a compact packaging for the spectrometer to work robustly against vibrations during transportation.

Author's Publication

Journal Articles

- **Si Chen**, Xinyu Liu, Nanshuo Wang, Qianshan Ding, Xianghong Wang, Xin Ge, En Bo, Xiaojun Yu, Honggang Yu, Chenjie Xu, and Linbo Liu. “Contrast of nuclei in stratified squamous epithelium in optical coherence tomography images at 800 nm”. *Journal of Biophotonics*. 2019;e201900073. (**Cover page publication attached as Appendix I; IF: 3.768**)
- **Si Chen**, Xin Ge, Xinyu Liu, Qianshan Ding, Nanshuo Wang, Xianghong Wang, Shufen Chen, Haitao Liang, Yunchao Deng, Qiaozhou Xiong, Guangming Ni, En Bo, Chenjie Xu, Honggang Yu, and Linbo Liu. “Understanding optical reflectance contrast for real-time characterization of epithelial precursor lesions”. *Bioengineering Translational Medicine*. 2019;e10137.
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- **Si Chen**, Xinyu Liu, Nanshuo Wang, Xianghong Wang, and Linbo Liu, “Seeing the Unseen using Micro-optical coherence tomography”. *CLEO Pacific Rim 2018*, Hong Kong. (oral presentation)
- **Si Chen**, Xinyu Liu, Nanshuo Wang, and Linbo Liu, “Evaluation of corneal endothelial cells using Micro Optical Coherence Tomography (μ OCT)”. *CLEO Pacific Rim 2017*, Singapore. (poster presentation)
- **Si Chen**, Xinyu Liu, Xianghong Wang, Xiaojun Yu, Nanshuo Wang, and Linbo Liu “Imaging microstructures of rat cornea using Micro Optical Coherence Tomography (μ OCT)”. *SPIE BiOS 2017*, San Francisco, CA, USA. (poster presentation)

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Appendix I