Development of lens-based probe designs and methods for depth-sensitive optical measurements

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Development of Lens-based Probe Designs and Methods for Depth-Sensitive Optical Measurements

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Abstract

This dissertation presents a series of studies on the development of non-contact lens based optical probe designs and data analysis methods for depth sensitive optical measurements, particularly Raman and fluorescence measurements, in skin pigmentary disorders.

A method of Biochemical Component Analysis was developed first to decompose and fully utilize the entire Raman spectra in elucidating the biochemical basis of Raman spectra which can be used for identification of tissue malignancy and evaluation of treatment outcome in pigmentary disorders. The algorithm was validated in a cell death model study using K562 cell lines.

Then, a novel cone shell illumination and detection configuration was introduced to enhance the depth sensitivity of conventional lens-based optical systems which employ a cone illumination and detection configuration. A Monte Carlo code was developed to simulate and investigate the depth sensitivity achieved by various combinations of illumination and detection configurations including both cone and cone shell configurations. Phantom experiments have been carried out to validate Monte Carlo modeling of fluorescence propagation in a two-layered turbid, epithelial tissue model.

To evaluate the cone shell illumination and detection configuration experimentally, an axicon lens-based probe was designed and constructed, which eliminated the need of altering probe-sample distance in performing depth measurements. The probe was evaluated for depth-sensitive optical measurements in terms of the sensitivity to the top and bottom layer in a two layered turbid skin phantom. It was found that the axicon lens-based probe has enhanced the sensitivity to

the bottom layer compared to that of an objective lens based probe with the cone configuration, and a larger range of sensitivity to either the top and bottom layer.

After that, we improved the spectra acquisition speed of the axicon lens based setup by incorporating five rings of collection fibers into the detection configuration. The acquisition speed of the probe was improved by five times in which optical spectra from five different depths can be collected simultaneously in a single measurement. The new setup got rid of the mechanical moving part consisting of two axicon lenses that are required to achieve depth sensitive measurements. The performance of this improved setup was evaluated in a fluorescence study using a two layered turbid tissue phantom.

In order to expand our depth sensitive optical probe from a point measurement system to an imaging system with a larger field of view, we demonstrated a multifocal noncontact setup capable of performing depth sensitive fluorescence imaging on a two-layered epithelial tissue model. The combination of a microlens array and a tunable lens enabled the depth of the multifocal plane to be conveniently adjusted without any mechanical movement of the imaging lens or sample. The performance of this improved setup was evaluated in a fluorescence imaging study by using a two-layered turbid tissue phantom and the result was further confirmed by spectral measurements.

In summary, the depth-sensitive optical probes and the data processing algorithm we developed are able to extract layer-specific information from turbid media, which is clinically useful for the diagnosis and treatment evaluation of skin pigmentary disorders. Further improvement of these techniques would help advance the use of optical spectroscopy in clinical settings.

Chapter 1 Overview

The objective of this research is to develop a depth selective optical measurement technique to characterize the layer dependent optical fingerprints of layered tissues. This method involves the development of a handheld optical probe for spectral acquisition, and a progressive estimation method and biochemical component analysis method for data analysis. The optical probe will be designed to detect Raman and fluorescence spectra from tissue layers at a range of depths below the tissue surface. The progressive estimation method will remove the contribution from a shallower layer to the spectrum measured from a deeper layer and the biochemical component analysis method will be used to quantitatively elucidate the changes in the biochemical constituents of tissue sample from the detected Raman spectra. The combination of these approaches will produce optical fingerprints specific to different layers.

Most medical spectroscopic applications, especially dermatology and biochemical identification in clinical diagnostics and treatments, involve the probing of human tissues. Human tissues are diffusely scattering media which are highly heterogeneous and often composed of multiple layers with different chemical make-up. The gold standard method to monitor subsurface tissue layers is the histopathology examination of tissue biopsies involving the excision of superficial tissue, which is a invasive procedure. A method capable of non-invasively extracting vital information about the chemical composition from each individual layers is highly desirable. The successful development of a handheld optical probe with depth resolved capability will allow non-invasive diagnosis of dermatologic disorders that originate underneath the skin. Depth selective optical measurement techniques will assist dermatologists in improving diagnostics and evaluating the treatment outcome of various skin disorders.

This dissertation is focused on the development of lens-based probe designs and methods for depth sensitive optical measurements in the skin. The following chapters are arranged in a logical sequence in which the significance of depth-sensitive optical measurements, the shortcomings of the current setup and our solutions are described.

Chapter 2 introduces the target tissue type, i.e. human pigmented skin lesions, the background of optical spectroscopy and its application in the detection of skin lesions. The importance of achieving depth sensitive optical measurements is discussed while the current state of art for depth sensitive optical measurement techniques is reviewed.

Chapter 3 describes a method of Biochemical Component Analysis (BCA) to decompose and fully utilize the entire Raman spectra in elucidating the biochemical basis of Raman spectra measured from biological samples. This method can be used for the identification of tissue malignancy and the evaluation of treatment outcome in various clinical applications including pigmentary disorders. The method was validated in a cell death model study using K562 cell lines in which cell spectra were decomposed into the contributions from several basic biochemical components including protein, lipid, nucleic acid and glycogen by employing a least squares regression algorithm to quantify the changes of cellular constituents in each death mode.

Chapter 4 investigates the depth profile of optical signals measured by a conventional cone configuration and a novel cone shell configuration that we proposed in transparent and diffusely scattering tissue phantoms. The result of this study may lead to a method that can separate the contribution of measured optical signal from each individual layer. A series of Raman experiments were conducted to obtain the performance of depth sensitive measurements using a microscope objective lens,

which employed a cone configuration, in transparent and turbid tissue phantoms. Then, the phantom experiments were repeated using the novel cone shell configuration and the depth sensitivities achieved by both the cone and cone shell configuration were compared. The advantage of the cone shell configuration in achieving better depth sensitivity compared to the cone configuration was demonstrated.

Chapter 5 describes the development of a Monte Carlo method to simulate the depth sensitivity achieved by various combinations of illumination and detection configurations including both cone and cone shell configurations implemented by convex lenses and axicon lenses. Phantom experiments have been carried out to validate Monte Carlo modeling of fluorescence in a two-layered epithelial tissue model. The method to implement a cone shell configuration in an axicon lens-based non-contact probe design is described in details. The data processing method for direct comparison between the experimental and numerical results, and the technical details of the Monte Carlo code which is capable of simulating fluorescence measured by different illumination and detection configurations are covered comprehensively.

Chapter 6 describes the implementation of the novel cone shell illumination and detection configuration in an axicon lens-based noncontact handheld probe design. The major advantage of the probe is that no alteration in the probe-sample distance is required to perform depth measurements, which will minimize inconsistent optical coupling in most other depth sensitive setups. The performance of the axicon lens-based probe and a microscope objective lens based probe was systematically evaluated for depth-sensitive fluorescence measurements in terms of the sensitivity to the top and bottom layer in a two layered turbid skin phantom.

Chapter 7 introduces a novel fiber assembly design that significantly improves the speed of spectra acquisition of the axicon lens-based setup described in Chapter 6.

The technical details of the fiber assembly and the modification to the optical setup are described. The performance of this improved setup was evaluated in a fluorescence study in term of the speed of acquisition and the sensitivity to the top and bottom layers by using a two-layered turbid tissue phantom. The acquisition speed of the probe was improved by five times in which optical spectra from five different depths can be collected simultaneously in a single measurement. Moreover, the new setup gets rid of the mechanical moving part including two axicon lenses that are originally required to achieve depth sensitive measurements, which will further improve the consistency of optical coupling and facilitate clinical uses.

Chapter 8 describes a multifocal noncontact setup capable of performing depth sensitive fluorescence imaging on a two-layered epithelial tissue model. The combination of a microlens array and a tunable lens enables the depth of multifocal plane to be conveniently adjusted without any mechanical movement of the imaging lens or sample. The performance of the setup was evaluated on a two-layered turbid tissue phantom in a fluorescence imaging study while the results were further confirmed by spectral measurements.

Chapter 9 gives the future directions in this multi-facet study and concludes with the major findings and novel inventions in this dissertation.

Chapter 2 Background

2.1 Pigmented Skin Lesion

Pigmented skin lesions or skin nevi refer to any cutaneous lesion clinically presenting as a tan to brown or black macule, patch, papule or plaque^{1, 2}. Pigmented skin lesions are very common while they can be born with or developed during the first two decade of a person's life. Without being noticed, each of us has a number of these skin lesions in different sizes and shapes, scattering all over our bodies. As most skin lesions are benign, we usually pay minute or no attention to these lesions unless they appear on cosmetically important regions such as the face, and are not pleasing to the eye.

The number of nevi on a person can be affected by several factors such as the demographic location, sun exposure frequency and genetic factor^{3, 4}. There is always probability for a nevus to mutate and turn cancerous. Generally, a large nevi count increases one's risk to develop a fatal melanoma. The average number of skin nevi in Caucasian population of age from 18 to 79 years old is 32 while the number can be as high as 400 in some people³. The average nevus count is found to be inversely proportional to the age as the number is higher, around 40, in the group of 18-30 years old and drops to around 10 in the group of 70 – 80 years old.³ In a recent study, Bataille et. al. show that there is positive correlation between nevi number and white cell telomere length, which suggests that those with higher numbers of pigmented lesions on their body may have an increased replicative potential or reduced senescence⁴. That means people losing moles quicker with age have shorter telomeres and suffer from relatively quicker aging. The finding of this report is so exciting

especially to the moley population who suffer slightly higher risk of melanoma as they enjoy a reduced rate of aging.

2.2 Neoplasia in Pigmented Skin Lesions

Figure 2.1 Anatomy of the skin, showing the epidermis, dermis, and subcutaneous tissue.
Adapted from <http://www.cancer.org>.

Our skin consists of three major layers, the epidermis, dermis and subcutaneous tissue. The epidermis is a stratified squamous epithelium. At the bottom of the epidermis, actively dividing basal cells differentiate into keratinocytes before they lose their nuclei, fuse into the squamous sheets and eventually get shed from the skin surface. Melanocytes reside at stratum basale, where they are interspersed between the basal cells and produce a pigment called melanin that is responsible for our skin colour. Pigmented skin lesions are the tumours of the basal cells, keratinocytes and melanocytes and can be classified into two categories: non-melanocytic and melanocytic. Generally, a lesion refers to the benign growth of cells within the tissue of origin. All lesions have probability to turn malignant. Non-melanocytic lesions arise from keratinocytes, basal cells and other types of cells in the epidermis including Langerhans cells and Merkel cells. Melanocytic lesions arise from melanocytes and

are commonly regarded as moles. A mole is formed when there is an increase in the melanin production or an increase in the proliferation of melanocytes that form a cell cluster.

2.2.1 Non-Melanocytic Pigmented Lesion and Skin Cancer

Non-melanocytic pigmented lesions are lesions found on the skin that contain pigment but are not melanocytes-derived. Benign non-melanocytic pigmented lesions that are frequently diagnosed are actinic keratoses, seborrheic keratoses, solar lentigines, dermatofibromas, pigmented Brown diseases and pigmented inflammatory conditions⁵. Fig. 2.2 shows an example of a seborrheic keratosis and solar lentigo. Seborrheic keratosis is a benign keratinocyte tumour which typically appears and grows rapidly in adults of age 30 and above. It contains varying amount of melanin pigment which can be uneven or asymmetric within the lesion. Even though it is harmless, it resembles melanoma, a more lethal type of skin cancer. Thus, accurate clinical diagnosis and identification of pigmented lesions is vital to ensure the effective clinical intervention of any skin complications.

Figure 2.2 Macroscopic picture of (A) a seborrheic keratosis adapted from Ref [6], and (B) a solar lentigo adapted from Ref [7].

There are two histological types of malignant non-melanocytic pigmented lesions named after the type of skin cells they arise from. Basal cell carcinoma (BCC) is the most common form of non-melanocytic skin cancers, accounting for more than 70% of all cutaneous malignant tumours in Asians, which arises from the basal cells at the lower layer of the epidermis⁸. The incidence of BCC in Singapore have been reported to be rising at a rate of 2.8% from 1968 to 1997⁹. BCC is commonly diagnosed in the sun-exposed area, usually on the neck or face. Pigmented BCC is commonly mistaken as a normal mole as it grows slowly and could take years to raise sufficient concern to be presented to the doctor. The common signs or symptoms of BCC are bleeding or scabbing sore but it can present in various forms from a shiny, pearly nodule to a red patch or a skin thickening, making it difficult to be visually diagnosed. BCC can be locally destructive but has little tendency to metastasize to the other part of the body, therefore is highly curable.

Figure 2.3 Macroscopic picture of a basal cell carcinoma adapted from Ref [6]

Squamous cell carcinoma (SCC) is the less common form of non-melanocytic skin cancer, representing 20-25% of the reported non-melanocytic skin cancer cases, which arises from squamous cells in the epidermis. SCC occurs mostly on the skin area which is regularly exposed to the sunlight or ultraviolet radiation especially on the

head and neck. Actinic keratosis is a precancerous skin neoplasm that may develop into SCC if not treated. The signs and symptoms of SCC are growing bump with rough and scaly surfaces and reddish patches. Fig. 2.4 shows a picture of a squamous cell carcinoma with rough and scaly surfaces and reddish patches. SCC rarely metastasizes but it is relatively more likely to spread than BCC.

Figure 2.4 Macroscopic picture of a squamous cell carcinoma adapted from Ref [10].

BCC and SCC are the most frequent malignant skin neoplasm and account for more than 95% of all skin cancer cases¹¹. Other less common forms of non melanoma skin cancers include Kaposi's sarcoma, cutaneous lymphoma, skin adnexal tumors, and Merkel cell carcinoma. Most non melanoma skin cancers are highly curable and can be treated effectively by various means including total excision, laser treatment and topical chemotherapy if identified in time.

2.2.2 Melanocytic Skin Cancer

Melanocytic skin cancer can be developed from melanocytic nevus, which is a common benign lesion originated from the melanocytes. Melanocytic nevus can be found easily on our bodies and is commonly regarded as mole. Some moles are present

at the time of birth while the rest are acquired in the early 20 years of a person's life. Benign moles are usually circular or oval, symmetrical in shapes with clear boundary and are not too large usually smaller than the size of a typical eraser. Most moles are harmless and possess only cosmetic concern if they grow on cosmetically important area such as the face. Fig. 2.5 shows a picture of blue nevus which is a common type of benign melanocytic pigmented lesion.

Figure 2.5 Macroscopic picture of a blue nevus adapted from Ref [6].

Even though rare, some melanocytic lesions can turn malignant and develop into lethal skin cancer. Melanocytic skin cancer or melanoma is the least common form of skin cancer which accounts for fewer than 2% of all skin cancer cases¹¹. However, melanoma is the most aggressive type of skin cancer due to its ability to metastasize early. Despite the low incidence rate, it is responsible for approximately 77% of deaths from all skin cancer cases¹¹. Melanoma is rare among the dark-skin population with a lifetime developing risk of around 0.1% in contrast to 2.4% in the white-skin population. According to American Cancer Society, the incidence rate of melanoma in the United States has been increasing in the past three decades while the incidence rate in the white population alone has increased by 2.7% from 2006 to 2010¹¹. In Singapore, the incidence rate of melanoma in Indian population with dark skin was

reported to be 0.2 per 100 000 while the incidence rate of melanoma in Chinese population with fairer skin was much higher reaching 0.5 per 100 000¹².

Malignant melanoma always starts from a benign nevus and is not easily distinguishable from a normal mole. Fig. 2.6 shows the main biological events in the progression of a melanoma. Melanoma can develop from an existing mole or by forming a new mole. Melanocytes in the stratum basale first actively proliferate to form a cluster of cells, seen as a mole on the skin surface. A benign nevus is usually round or oval, symmetric in shape, with clear boundary and even pigmentation throughout the nevus and it should not be larger than 5-6 mm in diameter¹³⁻¹⁵. A premalignant mole will further proliferate forming a larger dysplastic nevus with asymmetric shape, vague boundary and uneven pigmentation. The lesion will initially enter the radial-growth phase, where the size on the skin surface becomes significantly larger, before entering the vertical-growth phase, where the cancerous cells grow deeper into the skin and invade the dermis. At the final stage, the cancer cells reach the capillary and metastasize to other organs in the body.

Figure 2.6 Biologic events in the progression of melanoma adapted from Ref [16].

The survival rate of melanoma patient decreases with the disease stage while most of the melanoma patients are diagnosed at the very late stage. The early stage of melanoma progression resembles a typical mole with minimal or no discomfort that raises no concerns for detailed medical screening thus limiting the early detection of melanoma. Fig. 2.7 shows the pictures of an early melanoma and a benign mole. The early detection of melanoma is not easy as it is always mistaken for a benign nevus. Some benign moles can appear large on the skin surface but do not proliferate deep while some moles can appear tiny on the skin surface but invade deep into the skin and become highly malignant.

Figure 2.7 Macroscopic pictures of (A) a malignant melanoma; (B) a benign intradermal nevus.
Ref [7]

2.3 Current Procedure of Diagnosis and Treatment

The most common way of diagnosing a pigmented lesion when it is presented to a doctor is visual inspection. The doctor will first visually identify the type of lesion with the aid of a dermoscope or Wood's lamp by examining the surface morphology, structure and colour of the lesion. Then a suspicious lesion will be excised and histological examination will be performed on the excised specimen to accurately evaluate the type of lesion to decide whether further medical intervention is needed.

Generally, histological examination and total excision of lesions are the gold standard for diagnosing and treating pigmentary disorders¹⁷. However, the biopsy or total removal of every pigmented lesion is not clinically practical because it is not unusual that multiple skin lesions in a localized skin area are encountered. The excision procedure could also introduce the risk of scarring especially in the body parts that are cosmetically important such as the face. Furthermore, inappropriate surgery is frequently conducted as eighty percent of biopsies taken from suspected malignant skin lesions have been reported to be benign^{18, 19}. Therefore, visual inspection remains as the most practical procedure to diagnose a pigmented lesion.

One critical task in the diagnosis is the differentiation between a melanocytic lesion and a non-melanocytic lesion. A non-melanocytic lesion will be excised surgically or by laser eradication. Such treatment efficacy is high thus the recurrence rate is low. Once a melanocytic lesion is encountered, the malignancy of the lesion will be determined according to the ABCD rule¹³⁻¹⁵. A benign melanocytic mole does not require any medical intervention but a malignant melanocytic mole or melanoma has to be treated as early as possible. The disease progression of a melanoma has to be determined to decide on the most effective medical intervention method. Early melanoma can be cured by total excision or laser eradication with a high survival rate but for late stages of melanoma, the treatment method is more complicated as the cancerous cells have metastasized to other organs in the body.

Visual inspection is a subjective method which highly relies on the experience of the doctor. A benign mole can resemble a malignant one and results in unnecessary surgery while a premalignant mole may be mistaken as a benign one and causes critical delay in the subsequent medical intervention. Moreover, visual inspection of the tissue surface is rapid and non-invasive but it can only look at the structures and

patterns of pigments in a planar view²⁰. Extra information of the lesion such as the thickness and depth are vital to help in improving the doctor's diagnostic accuracy. Tissue biopsy and histological examination is the gold standard for accurate pigmented lesion diagnosis that could provide these information but they are invasive, labour intensive and time consuming. Therefore, it is crucial to develop a technique to conveniently probe clinically suspicious pigmentary disorders in a non-invasive way to assist in diagnosis and treatment planning to reduce the clinical complication from pigmented lesions.

2.4 Optical Spectroscopy and Its Application in Detection of Skin Neoplasia

Dermatology and many other medical applications involve the examination of human tissues for biochemical identification in clinical diagnostics and treatment. Human tissues are diffusely scattering media which are highly heterogeneous. For the purpose of modeling, the skin or other epithelial tissue is often treated as multiple layers each with different chemical make-up. A method capable of non-invasively extracting vital information about the morphological, structural and chemical information from each individual layer is highly desirable.

Recently, optical techniques have been widely explored in the clinical evaluation of skin diseases due to the non-invasive nature of the techniques and the existence of a wealth of biological molecules that interact with light to generate rich biochemical information²¹⁻²³. Because of the non-invasiveness of light, there is virtually no limit on the number of measurement repetitions while a doctor can rapidly and conveniently probe on any suspicious location as needed. The development of sensitive detectors and fiber optic technology has improved these techniques in terms of speed, sensitivity and portability, allowing real-time optical spectral measurements in the clinical setting

to aid medical diagnosis. Diffuse reflectance and fluorescence spectroscopy are sensitive to changes in the optical properties and autofluorescence properties in the skin tissue. The changes in tissue optical properties due to the variation of cell density and size from normal tissue to cancer and the presence of endogenous fluorophores relevant to the pathology state of skin tissue have made reflectance and fluorescence spectroscopy important tools in the detection of skin neoplasia.

Diffuse reflectance spectroscopy is able to translate information about the subcellular tissue morphology as well as the endogenous chromophore content such as hemoglobin and melanin²⁴ into spectral features. The extracted diagnostic information can be compared with results from the histological examination. The strong correlation between the two sets of results suggests that diffuse reflectance spectroscopy is a potential non-invasive alternative for cancer diagnosis. Volynskaya et. al.²⁵ and Zhu et. al.²⁶ demonstrated the use of diffuse reflectance spectroscopy in breast cancer diagnosis, which has also been used in other *in vivo* research areas including cervical²⁷ and oral oncology²⁸.

Fluorescence spectroscopy has been widely explored for the detection of precancers and cancers of various tissues. This is an attractive technique as it is capable of rapidly and non-invasively quantifying biochemical and morphological changes in human tissues due to the rich endogenous fluorophore contents in human tissues that contain vital diagnostic information for the identification of tissue malignancy. Ebert et. al.²⁹ performed fluorescence imaging on malignant melanomas and nevi and Leeuw et. al.³⁰,³¹ has used fluorescence spectroscopy in the detection of non melanoma skin cancers at the early stage. Fluorescence spectroscopy has also been explored in other cancer detection such as lung cancer³², colorectal cancer³³ and gastrointestinal cancer³⁴.

In particular, Raman spectroscopy is superior to other optical techniques in term of chemical specificity³⁵. Skin tissues contain many Raman active biomolecules such as proteins, lipids, nucleic acids, polysaccharides, various skin pigments such as melanin³⁶. These components emit Raman signals that carry rich biochemical information about the skin tissue in bulk, which can be recovered from a detected Raman spectrum to obtain their individual contributions for the clinical evaluation of diseases and the monitoring of the treatment outcome. Thus Raman techniques have been explored in various skin research areas such as the in vivo measurements of carotenoid concentration of skin²¹, detection of melanoma²² and non-melanoma skin cancer²³.

2.5 Depth Sensitive Optical Measurement for Skin Diagnosis

Most skin neoplasia as well as other pathological processes develop from the subsurface region of the skin³⁷. Disease diagnosis based on the visual inspection of the pathological presentations or symptoms on the skin surface are subjective and highly depend on the experience of the doctors. Vital diagnostic information for the accurate identification of diseases is usually located underneath the surface and its depth distribution is known to be related to disease progression³⁸. In addition to the qualitative pathological appearance available from the tissue surface, this subsurface diagnostic information will not only help the doctor improve the diagnostic accuracy but also provide vital input for more effective treatment planning, for example, in the determination of optimal laser therapy parameters to achieve desirable laser treatment outcome. Currently, the most accurate way to obtain this information is by histological examination. However, this gold standard method is invasive, time consuming and labor intensive.

Even though optical spectroscopic measurements are fast and non-invasive, the accurate retrieval of the depth-specific diagnostic information is complicated by the heterogeneous nature of human tissues. The optical signal measured from a tissue is the result of averaging from a larger tissue volume that mixes information from the region of interest and the surrounding tissues region, especially from the overlaying layers. Thus, researchers have been working towards different depth sensitive optical measurement methods that are highly desirable for the accurate extraction of useful depth-specific optical information from human tissues.

In fluorescence spectroscopy, depth sensitive measurements in turbid media have previously been demonstrated using fiber-optic probe geometries with varying source-detector separation³⁹, aperture diameter⁴⁰, and tilt angle of illumination and collection fibers⁴¹. However, a fiber-optic based probe requires physical contact with the sample surface and a past study has shown that the inconsistent pressure exerted on biological tissues can cause spectral distortion that severely affects the diagnostic accuracy of this technique⁴². Besides that, the contact between the probe and the patient's skin especially on infectious sites may cause probe contamination and elevate the risk of disease transmission across patients.

A design similar to the probe design with varying source-detector separation in fluorescence spectroscopy, Spatially Offset Raman Spectroscopy (SORS), has been developed to retrieve Raman spectra from subsurface layers at different depths in scattering media⁴³. Schulmerich et. al⁴⁴ has demonstrated the efficacy of this technique in the transcutaneous measurements of bones. In SORS, the relative Raman intensity ratio of the bottom layer to the overlying layer can be increased by enlarging the spatial offset between the source fiber and the detector fibers. The signal from the bottom layer diminishes much slower than the upper layer because Raman photons generated from the deeper layer are more likely to migrate laterally before exiting

from the surface⁴³. Because both fibers are perpendicular to the tissue surface and light scattering in tissue is highly forwarding, the examined tissue volume is quite deep even for a small source-detector separation. The increase in light path causes significant attenuation in Raman signal intensity due to photon diffusion process and light absorption. This poses a weakness of the technique, i.e. the fact that the Raman peaks from various cutaneous layers, are inherently weak and highly overlapping. A highly sensitive detection system is required to achieve a high signal to noise ratio so that the Raman contribution of any individual biochemical component is distinguishable from others. Moreover, the inconsistent probe-sample contact can still induce uncertainty in measured spectra as in fluorescence spectroscopy. This problem could be even severer given that the Raman signal is much weaker.

Being a non-contact lens based modality, confocal microscopy has attracted significant attention in depth selective measurements. One such example is confocal Raman spectroscopy, which combines confocal microscopy technique with Raman spectroscopy to offer high-resolution optical sectioning and depth profiling^{45, 46}. This technique allows the detection of Raman signals only from the plane of interest by rejecting out-of-focus light⁴⁷. Casper et al. has used this technique to derive the depth resolved concentration profiles of water and natural moisturizing factors in stratum corneum for a depth range of 0 to 250 μm in human skin in vivo⁴⁶. Wang et al.⁴⁵ used confocal Raman technique to differentiate subcutaneous tumor from normal skin in a depth range of 0 to 40 μm beneath the surface. Lieber et al.⁴⁸ developed a handheld confocal Raman probe for non-melanoma skin cancer diagnosis. However, a recent study showed that Raman photons in a diffusely scattering sample were obtained from an extended illuminated volume instead of a well defined focal point⁴⁹. The signals from planes well above and beneath the focal point contributed significantly to the

Raman even after the removal of surface aberration and refraction effects by using oil immersion objective lenses⁴⁹.

In short, the major limitations of the current methods for depth sensitive measurements include the following: 1. having to contact with sample surface in a fiber-optic based setup; 2. requiring the movement of the imaging lens or sample in a non-contact lens-based setup; 3. being subject to interference from out-of-focus signals; 4. limited depth sensitivity to the subsurface layer. In the following chapters, a non-contact lens based handheld probe design for depth sensitive optical measurements in skin tissues and associated data processing methods were pursued in an attempt to overcome the above limitations of the current methods. A novel cone shell illumination and detection geometry was introduced to improve the depth sensitivity of current art which employs the conventional cone geometry. A portable axicon lens-based probe was constructed to implement the illumination and detection geometry and its depth sensitivity in fluorescence measurements was evaluated numerically and experimentally using a two-layered turbid tissue model. The performance of the probe was then improved with the incorporation of a collection fiber assembly which allows spectra to be recorded from five depths simultaneously in one time of data acquisition and effectively improve the speed of data acquisition of the proposed system by five times. Furthermore, a microlens array was employed in the effort to improve the speed of acquisition by expanding the depth sensitive measurements to a multi-focal imaging setup in a large field of view. The system was evaluated systematically on a turbid tissue phantom in a fluorescence study. On the other hand, the depth profile of optical signals measured by the cone and cone shell configurations was investigated in layered tissue phantoms as demonstrated in Chapter 4, which may lead to a method that can separate optical signals from different tissue layers.

Chapter 3 Development of Biochemical Component Analysis Method for Raman Spectroscopy in a Cell Death Study

[Copyright permission from: Y. H. Ong, M. Lim and Q. Liu, "Comparison of principal component analysis and biochemical component analysis in Raman spectroscopy for the discrimination of apoptosis and necrosis in K562 leukemia cells," Optics Express 20(20), 22158-22171 (2012)]

3.1 Introduction

Raman technique has become increasingly popular in biomedical science and engineering due to several of its advantages. First of all, Raman requires little or no sample preparation thus allowing non-destructive measurements of a sample. A biological sample, for example a cell, can be measured over a long period of time without being damaged. Most biological samples are in aqueous forms, and Raman is superior over many other spectroscopic techniques in measuring from aqueous solutions since it does not suffer from the large water absorption effects. Furthermore, the intensity of spectral features in solution is directly proportional to the concentration of particular species⁵⁰. Thus, useful biochemical information about the constituents of the sample as well as the concentration or amount of each component can be extracted from Raman spectra. Owing to these advantages and its high chemical specificity, Raman has become a very powerful tool for biological analysis and chemical monitoring in various fields, including biomedical applications.

A typical Raman spectrum is generated by plotting the light intensity against Raman shift in the unit of wavenumber. The Raman shifts are proportional to the vibrational energies or characteristic frequencies of molecular bonds or functional groups. Thus by referring to a lookup table of Raman peak assignments such as the example in Table 3.1, the chemical composition of a sample can be identified.

Table 3.1 Peak Assignment for Raman Spectra of A549 Cells. Table adapted partly from Ref [51]

Peak Position (cm ⁻¹)	Assignment			
	Nucleic acids	Proteins	Lipids	Carbohydrates
1659		Amide I α helix	C=C str.	
1460		CH def.	CH def.	CH def.
1449		CH def.	CH def.	
1342	A,G	CH def.		
1258		Amide III β sheet	=CH def.	
1095	PO ₂ ⁻ str.	Chain C-C str.	C-C str.	
1013	C-O deoxyrib.			C-O str.
828	O-P-O str.	Ring br. Tyr		
728	A	Ring br. Trp	C-N head group	

In an aqueous solution, the unknown concentration of a component can be determined by comparing the peak Raman intensity to the peak value of a reference solution with known concentration. This can only be done when a band is distinct and does not overlap with bands from other components. Biological samples are aqueous solutions that are mainly made up of water, proteins, lipids, carbohydrates and nucleic acids. Water has a low Raman scattering cross section and has a characteristic band at high wavenumbers which do not interfere with other components' spectra. However, the Raman spectra of other basic biochemical components in biological samples are highly overlapping in the region of 600 cm⁻¹ to 1800 cm⁻¹. Table 1 shows that a characteristic bond can be found in multiple bio-macromolecules. For example, CH deformation bonds have Raman peaks around 1300-1500 cm⁻¹ and are abundant in proteins, lipids and carbohydrates. Besides that, C-O bonds, which can be found in DNA and carbohydrates, have prominent Raman peak at 1013 cm⁻¹.

Most skin studies with Raman spectroscopic technique used the change in Raman peak intensity to estimate the variation in the amount of biochemical component^{48, 52, 53}. In this case, an inherent assumption was made that the intensity of peak assigned to a particular biochemical component is exclusive of the contribution from other component. This may introduce inaccuracies to the result as there are bonds from other components that have identical Raman shifts under the same excitation even though the intensity may be lower. Biological samples contain complex mixtures of biochemical components, including proteins, carbohydrates, lipids and nucleic acids, which are enclosed within cell membranes. Understanding various cellular processes requires the identification of substances inside living cells. These biochemical components are organic compounds that emit Raman signatures in the low wavenumber region (600 cm^{-1} to 1800 cm^{-1}). Direct peak intensity comparison method is not suitable to analyze the changes in the amount of these components because their Raman spectra are highly overlapping. Furthermore, the assignment of each prominent peak or band to one biochemical component during intensity comparison could cause missing valuable information about the cell composition. Thus, there is a need to develop a data analysis method that fully utilizes the Raman spectrum of a biological sample to elucidate the biochemical changes underlying each biological process.

In response to the issues discussed above, we developed a spectral decomposition method to estimate the individual contribution of the basis constituents in biological tissues by taking into account the full Raman spectrum measured in order to prevent missing any useful spectral information. This spectral decomposition algorithm, regarded as Biochemical Component Analysis (BCA) for the rest of this report, will allow for accurate quantification of biochemical components in a biological sample even when there is overlapping Raman bands between these

components. As for the purpose of development and demonstration of this algorithm, a cell death model was employed where the changes in the biochemical compositions of live, apoptotic and necrotic cells will be elucidated quantitatively. The reason a cell death model was chosen is because the biological events and biochemical changes associated with each type of cell death have been well documented and those reports serve as gold standard for our results to be compared to. To facilitate the interpretation of the results of spectral decomposition, the biochemical changes as well as the morphological changes and mechanisms underlying each type of cell death will be briefly discussed in the following section. The importance of cell death research and the advantages of Raman spectroscopy over the other techniques will also be discussed.

3.2 Cell Death and Its Importance

Cell death is essential in maintaining homeostasis in the biological system. Two distinct modes of cell death, apoptosis and necrosis, differ in the morphology, incidence and mechanism.⁵⁴ Apoptosis is an active and genetically regulated way of cell death and is commonly regarded as programmed cell death. Cells of different lineages commit suicide through a common series of regulated cascade of events which give rise to profound hallmark changes. Morphologically, apoptotic cell shows cell shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies. Common biochemical events found in apoptotic cells are alteration of protein level, changes in membrane potential, DNA fragmentation and RNA biosynthesis.^{55, 56} Changes on membrane surface act as signals and apoptotic cells are rapidly recognized and engulfed by neighboring cells or phagocytes. Cellular leakage is prevented and no inflammatory response is induced.⁵⁷

Necrosis is normally regarded as accidental death. This mode of cell death is not genetically regulated and occurs when a cell is severely injured or induced by extremes in external environment such as hypoxia, membrane active toxicants, and respiratory poisons.⁵⁸ When irreversible changes occurred to the nucleus or cytoplasm, necrosis is signaled. Cell membrane integrity is lost and results in loss of membrane concentration gradient. In contrast to apoptotic cells, a necrotic cell and its organelles swell and DNA is randomly degraded. At the later stages of necrosis, plasma membrane ruptures and lysosomal enzymes leaks out causing inflammatory response in the surrounding tissues.⁵⁹⁻⁶¹

The study of cell death *in vitro* is vital to understand the molecular mechanism underlying each mode of cell death and unveil new strategies in drug development and treatment prognosis for several pathological states. Intense researches on apoptosis over the past twenty years have expanded our knowledge on its role in various normal and pathological processes. Apoptosis has found its significance in many disease prognosis and treatment development especially in cancers and tumors.^{62, 63} Most commonly used drugs for tumor and cancer therapy nowadays target at the apoptotic pathway to wipe out cancerous cells with minimal inflammation response. However, some cancerous cells may acquire resistance to induce apoptosis by random mutations in the apoptotic protein machinery.⁶⁴

Complete cancer removal is hard to achieve with single drug treatment because cells with that particular drug resistance may survive and remain dormant in the host. The recurrence of the disease will be more critical due to the selective pressure on the cancerous cell population that chose the most resistant traits. Thus, a new way to target these apoptosis resistant cells is important to compliment current drugs for better treatment outcome. Necrosis, comparatively, was not the main focus of cell death

studies for treatment development because it is the non-favorable way of cell death, resulting in inflammatory response in the surrounding tissue. However, recent research have shown that, instead of occurring during pathological events, necrotic cell deaths were found consistently existing in several normal physiological processes.⁶⁵⁻⁶⁷ Studies have since then revealed that the disturbance of the fine balance between apoptosis and necrosis may contribute to the development of various diseases.⁶⁸ Although most of the commonly used anti-cancer compounds are apoptosis inductive, there are still many drugs that induce necrosis.⁶⁹ They are important in tumors resistant to apoptotic drugs and to those that have mutation in their apoptotic pathways. Some studies also suggested that necrosis can potentially have a wider range of action than apoptosis since it is not dependant on a handful of apoptotic protein.⁷⁰ Thus the development of necrosis inducing drugs is important as complement or even alternative compounds as chemotherapeutic agents.

There are many well established biochemical techniques to assess the efficacies of these drugs *in vitro* by studying cell death, such as DNA electrophoresis, comet electrophoresis, TUNEL and flow cytometry. Most of these methods are based on the plasma membrane permeability where they measure the cellular retention or exclusion of fluorescent dye as an indicator of cell viability and death mode. They require cell staining with fluorescence dye, which is the main limitation in *in vivo* studies. Besides that, the introduction of dye may alter or damage the cells and causes unwanted distortion in the results. Furthermore, these techniques only provide single snapshot for the result due to the photobleaching of fluorescein but cell death is a time dependent process where there is yet any well characterized checkpoint for the event. Qualitative studies of cell death based on fluorescence images are even complicated by

the heterogeneity of apoptotic cells in vitro, where they can switch to the secondary necrosis pathway in the absence of phagocytes.

3.3 Raman Spectroscopy in Cell Death Research

Raman spectroscopy is a laser-based spectroscopic technique that is capable of measuring the inelastic scattering of photons induced by the intrinsic molecular bonds present in a sample. It has the ability to obtain rich biochemical information from single cells without the need of staining or labeling thus this technique has been explored in a variety of cell studies. For example, Raman spectroscopy has been employed to study the cellular components of individual living cells⁷¹⁻⁷³, to monitor the differentiation of human embryonic stem cells into their cardiac derivative⁷⁴, and to monitor hydroxyapatite in the differentiation of human mesenchymal stem cells into osteoblasts.⁷⁵ Raman spectroscopy is also used to monitor cell cycle and proliferation^{76,77}, and to distinguish between healthy and cancerous cells.^{78,79}

Several studies have employed Raman spectroscopy technique to investigate the Raman spectra pattern of apoptotic or necrotic cells. Yao et al. distinguished between live and apoptotic gastric carcinoma cells by comparing the intensities of several Raman peaks of the cell spectra. Significant reduction was observed at the major vibrational bands generated by protein, DNA and lipids⁸⁰. Notingher et al. has studied the Raman spectra of live and necrotic human lung epithelial cells and decreases in nucleic acids and protein were reported.⁵¹ Besides direct comparison between intensities of Raman peaks assigned to specific biochemical components, Raman imaging of biochemical distribution in cell was also demonstrated in previous studies. By careful selection of Raman peaks assigned solely to the characteristic bonding of a particular component, Uzunbajakava et al. has mapped and visualized the

distribution of DNA and protein in single apoptotic HeLa cells by using DNA peak at 788 cm^{-1} (cytosine and uracil ring stretching modes) and protein peak at 1451 cm^{-1} (CH_2/CH_3 bending vibrations).⁸¹ Recently, Zoladek et al. demonstrated the time-course Raman imaging of live and apoptotic human breast cancer cells over 6 hours by using 788 cm^{-1} (DNA) and 1659 cm^{-1} (lipid) bands.⁸²

All the previous studies presented above performed visual inspection on cells spectra by assuming that the peaks under studied were solely contributed by the intrinsic bonds of the assigned biochemical components. This assumption may introduce inaccuracies to the result as spectra of basic biochemical components of cell are highly overlapping. Several components may contribute to the same peaks at the same excitation wavelength and visual inspection of these peaks usually involves guessing to determine to which biochemical components that these changes correspond. Changes in specific Raman bands thus provide little quantitative understanding of how the relative amounts of basic biochemical components change across each cell type.

Due to the drawbacks of classical visual inspection method, chemometrics methods emerged to be a better approach for implementing spectral diagnosis by utilizing the entire Raman spectral. By using statistical or mathematical techniques, the essence of information present in the spectral data can be highlighted and represented in a matrix with reduced dimension for easier quantitative interpretation. Commonly used methods include Biochemical Component Analysis (BCA)⁸³⁻⁸⁷, and principal component analysis (PCA)⁸⁸⁻⁹¹. BCA method uses least square regression, by assuming the sample's spectrum is the linear summation of all basic components' spectra, to estimate the contribution of each component. This method is named differently in the literature, which includes spectral deconvolution, basis spectral

analysis method⁸³, direct classical least squares method (DCLS)⁸⁴, and spectral fitting method^{86, 87}. For the purpose of convenience, this method will be regarded as Biochemical Component Analysis (BCA) in this report. Kunapareddy et al. has demonstrated this method by fitting the basis spectra of protein, lipid, RNA, DNA and glycogen to full cell spectra to estimate the biochemical changes in necrotic human malignant melanoma cell (MEL-28). They reported a decrease in the relative amount of lipid and RNA in necrotic cells, and observed an increase in the relative amount of protein⁸³. This technique has also been reported earlier at the tissue level in a study of breast cancer diagnosis⁹². The quantification of biochemical changes can provide useful information of the structural and pathological states of cells and help to classify different cell and disease types. While this is a fast and quantitative approach, BCA requires prior knowledge of the pure constituents of the sample to supply an explicit background model to the algorithm. Moreover, spectral fitting algorithm in BCA is also sensitive to changes in the background spectrum as it cannot adapt to peak shifts and alterations in the relative intensities of peaks⁹³.

The principal component analysis (PCA) method is a non-parametric method that does not require an explicit background model. PCA performed mathematical decomposition of the spectral data that reduce the data dimensions of a highly complex chemical system to a smaller number of scores and principal components (PCs) or loadings that effectively carries all the important information of the spectra⁹⁰. Classification of spectral data can be easily done by choosing different combinations of PCs to build a new coordinate system. PCA is widely used in Raman spectroscopy studies for pathological classification, such as to discriminate between Barrett's and normal epithelium⁹⁴, to differentiate adenomatous from hyperplastic polyps of the colon⁹⁵ and also to classify T and B lymphocytes of normal and leukemic patients⁹⁶.

In a cell death study, Yao et al. has demonstrated the use of PCA method to distinguish between live and apoptotic human gastric cancer cells⁸⁰. However, while useful as a classification method, PCA does not reveal physically or chemically interpretable information of the sample. It provides only abstract information representing entire features broadly distributed in the data and is unable to explicitly quantify the biochemical changes in a biological sample^{88, 91}.

In this work, we will first compare the efficacies of classical visual inspection and BCA methods in extracting features from Raman spectra of live, apoptotic and necrotic human chronic myelogenous leukemia cells (K562 cell line). In BCA study, the Raman spectra of cells will be decomposed to estimate the contribution from basic cellular constituents, such as proteins, nucleic acids, lipids and polysaccharides, to quantify the biochemical differences among live, apoptotic and necrotic leukemic cells. These results will be validated against the literature. Then, PCA will be performed on the spectral data to yield principal components and their scores. Finally, a linear discriminant analysis (LDA) method^{97, 98} will be employed to distinguish cell death modes based on the features extracted from both BCA and PCA to compare their classification accuracies. Furthermore, the principal components from PCA will be decomposed by using BCA method in an attempt to interpret the biochemical information behind them.

Single cell Raman measurements require the excitation laser spot to be accurately focused within a cell. Cell trapping is important when measuring Raman signal from suspension cells to ensure that light remains focused on the cell throughout the measurement. The movement of cells and the evaporation of buffer solution during data acquisition will introduce inaccuracies to the Raman spectrum obtained. Most of the previous studies about cell death have concentrated on adherent cells, such as

epithelial cells^{51, 80-83, 99}, where cells typically stay anchored during measurements thus easy to keep cells on focus. Although there were research groups employing different methods such as air-drying cells on CaF₂ slides¹⁰⁰, micropipette¹⁰¹, chemical methods¹⁰¹ or optical trapping method¹⁰²⁻¹⁰⁴ to immobilize suspension cells on planar surfaces during Raman measurement, these methods may induce undesirable perturbations to cellular biochemical reaction. Air drying¹⁰⁰ of cells may cause changes to Raman spectra due to deformation in biomolecules since most of the cellular components are water soluble. Chemical immobilization¹⁰¹ may disturb cell homeostasis by changing the microenvironment of living cells. Micropipette tends to induce changes in cell membrane due to the surface effect that could alter the normal shape of cells¹⁰¹. Optical trapping method using near infrared lasers can minimize the physical and chemical perturbation to the trapped cell¹⁰⁵. However, long handling time in a small focal volume may result in the heating of water based buffer due to the absorption of laser energy, which can cause damage to the cells¹⁰⁶. To address the issue mentioned, we demonstrated a simple method to minimize cell movement and to prevent the evaporation of buffer solution during the measurement of Raman spectra from individual leukemic cells in suspension by creating a shallow well with aluminum sheet. The detail of this method will be discussed in detail in the following section.

3.4 Materials and Methods

3.4.1 Sample Preparation and Drug Treatment

Human chronic myelogenous leukemia cells (K562 cell line) were purchased from American Type Culture Collection (Manassas, VA, US). K562 cells were

cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum, and were incubated in the incubator at 37°C and with 5% CO₂. Cultures were maintained by the addition or replacement of fresh medium every 2-3 days to maintain the cell density between 10⁵ and 10⁶ cells per ml. Cells were then transferred to a 6-well culture plate at cell density of 10⁵ per ml and were incubated at 37°C and with 5% CO₂ for 24 hours. Cytosine arabinoside (Sigma Aldrich, Singapore) was then added to two wells to reach a final concentration of 300 µM to induce apoptosis. Triton X-100 was added to another two wells at a concentration of 100 µM to induce necrosis. This concentration will induce damage to cell membrane without completely lysing it in the first 24 hours upon induction⁹⁹. The other two wells were used as the control group without drug treatment. All groups were incubated for another 72 hours at 37°C and with 5% CO₂. Cells from the control group and the treated groups, were washed twice, rinsed and immersed in phosphate-buffered saline (PBS). A small cell sample from each treated group was taken to validate the occurrence of apoptosis and necrosis while the remaining cells were left unstained for Raman measurements. The apoptosis of the cells treated with cytosine arabinoside were tested and validated by applying a dye, Hoechst 33258. Necrotic cells treated with Triton X-100 were validated by using Trypan blue dye, which is a membrane permeation assay.

3.4.2 Evaluation of Apoptosis and Necrosis

Apoptotic cell death was evaluated by Hoechst 33258, a blue fluorescent dye that stains nucleic acids. A cell sample taken from the well plate where Cytosine arabinoside was applied was first centrifuged and resuspended in PBS solution to wash away culture medium. Hoechst dye was added to the sample to reach a final concentration of 2 µg/ml. The mixture was incubated for 15 minutes at 37°C before being washed twice and immersed in PBS solution. The sample was observed under a

fluorescence microscope with 350-nm excitation light. Apoptotic cells were confirmed by nuclear condensation and fragmentation while the nucleolus and chromatin of control cells remained intact.

To evaluate cell death due to necrosis, 10 μl of 0.4 % (w/v) Trypan blue solution was added to 10 μl of the cell sample. The mixture was left for 5 minutes in room temperature before the observation under optical microscope. Necrotic cells were stained blue while live and apoptotic cells remained unstained as their plasma membrane remained intact.

3.4.3 Raman Measurements

Raman spectra were measured using a micro-Raman spectrometer system (inVia, Renishaw, UK) coupled to a microscope (Alpha 300, WITec, Germany) in a backscattering geometry. A Czerny-Turner type spectrograph ($f = 250 \text{ mm}$) equipped with a holographic grating (1800 groove/mm) and a RemCam CCD detector (inVia, Renishaw, UK), which yields a spectral resolution of 2 cm^{-1} , were selected for this study. A 785-nm diode laser (about 50 mW on the sample) was used for excitation and the illumination time was 60 seconds. It was reported in another study¹⁰⁷ that no visible effect on cell viability was observed when cells were illuminated by a laser at the same wavelength with the identical power for 120 seconds. Similarly, no visible effect on cell viability was observed in this study either during or after each measurement. The laser spot of around $1.6 \mu\text{m}$ in diameter was formed on individual cells by a microscope objective lens (50x, NA=0.6, Leica). The cell sample was prepared on an aluminum substrate to achieve minimal Raman and fluorescence background and covered by a thin quartz cover slip as shown in Fig. 3.1 to reduce the distortion on focusing due to evaporation. A small well was created using a cellophane

tape underneath aluminum sheet that wrapped around a glass slide to limit the movement of cells during focusing adjustment, while keeping the sample in suspension. This well design also helped reduce the pressure on the cell exerted by the cover slip thus keeping cell morphology unchanged.

Figure 3.1 Cross sectional view of the sample prepared in a small well created on an aluminum substrate.

3.4.4 Data Analysis

Twenty cells from each group were measured over a range of 600 cm^{-1} to 1800 cm^{-1} to obtain average spectra representing each cell type (live, apoptotic and necrotic cells). The background spectrum was measured from PBS on the aluminum substrate as in Fig. 3.1 and subsequently subtracted from cell spectra. Data processing was performed using MATLAB (Version 7.6, MathWorks, Natick, MA, US). Firstly, the narrow spikes caused by cosmic rays were removed. Then, the broad and slowly varying fluorescence background was estimated by using the fifth order polynomial fitting and subtracted. Afterwards, each spectrum was smoothed using a Savitzky-Golay smoothing algorithm before the subsequent analysis was performed.

PCA was first performed on measured Raman spectra, using *princomp* function in MATLAB. PCA is a statistical analysis method which can reduce the dimension of the data while accounting for most of the variance in the original data. Kruskal-Wallis one-way analysis of variance was performed on the scores of the first ten principal

components to determine which PC has significant differences in the mean of scores among three groups of cells. Two-dimensional plots were constructed with different combination of scores for the first three principal components and a three-dimensional plot was also constructed with the three sets of scores.

For BCA, the Raman spectra of pure basic biochemical components, including actin, albumin, triolein, phosphatidylcholine, DNA, RNA, and glycogen, were measured with the same configuration as in cell measurements. These spectra were normalized by dividing the intensity at each wavenumber by the maximum intensity of each spectrum and the normalized spectra were used in BCA. All components were purchased from Sigma Aldrich, Singapore and used without further purification. These components were chosen to represent the major biochemical groups in cellular constituents, which include proteins, lipids, polysaccharides, and nucleic acids. The intensities of the component spectra were assumed to be linearly proportional to their concentrations and the cell spectra were the linear combination of these basic component spectra at various concentrations. To find out the contribution of each component to the cell spectra, a least square regression method was employed by using a nonlinear curve fitting function, *lsqcurvefit*, in MATLAB with the option of the trust-region-reflective algorithm. This function finds the coefficients of all basic component spectra that best fit the cell spectra. These coefficients reflect the relative amount of biochemical components in cells. Wilcoxon signed-rank test was performed to evaluate the statistical significance of difference in the amount of biochemical components between different groups of cells. The principle of the spectral decomposition algorithm will be illustrated in the following paragraph.

The spectral decomposition algorithm or BCA is based on the following assumption, i.e. the Raman spectrum of a biological sample can be treated as the linear

summation of the Raman spectra of basic biochemical components with different weightings. This assumption is expressed in a mathematical expression as follows:

$$y_j = a_1x_{1,j} + a_2x_{2,j} + a_3x_{3,j} \dots + a_ix_{i,j} \quad (3-1)$$

where,

y_j = Intensity of the sample spectrum at the j -th wavenumber;

$x_{i,j}$ = Intensity in the spectrum of the i -th biochemical component at the j -th wavenumber;

a_i = Coefficient/weighting of the i -th biochemical component;

In this cell study, actin, albumin, triolein, phosphatidylcholine, DNA, RNA, and glycogen, have been identified to be seven major biochemical components contributing most to the cell spectra. Thus, the equation can be written as:

$$y_j = \sum_{i=1}^7 \sum_{j=1}^n a_ix_{i,j} \quad (3-2)$$

where,

n is the number of wavenumbers in Raman spectra.

A least square curve fitting algorithm is used to find the coefficient values for all basic components' spectra that best fit the equation and returns the least residue as required by the following expression:

$$\min_a \sum_{i=1}^7 \sum_{j=1}^n (a_ix_{i,j} - y_j)^2 \quad (3-3)$$

where,

n = length of x and y

The coefficients that best fit the equation, which is set to be in positive scalar numbers, can be calculated given the x and y data acquired from Raman measurements as described earlier.

After performing BCA and PCA on the cell spectra, we compared the performance of cell death classification using the features extracted from the two methods. Linear discriminant analysis (LDA) classification models were built using the fitting coefficients of biochemical components from BCA and principal components from PCA. A leave-one-out cross validation method¹⁰⁸ was employed to train the classifier. The performance of these models was compared in term of accuracy in classifying different cell death modes. Furthermore, the first ten principal components from PCA were decomposed using BCA in an attempt to interpret those principal components showing significant differences among different groups.

3.5 Results

Fig. 3.2 shows the Raman spectra of seven selected basic biochemical components. Note that the spectra of each individual biochemical component were vertically segregated for clarity. These basic components are used to represent the four major organic bio-macromolecules in cells, i.e. proteins, lipids, nucleic acids, and polysaccharides. Among the seven components, actin and albumin represent proteins, triolein and phosphatidylcholine represent lipids, DNA and RNA are nucleic acids and glycogen represents polysaccharides. It is noted that the Raman spectra of actin, triolein and DNA are quite similar to those of albumin, phosphatidylcholine and RNA, respectively. The selection of these components will be discussed in detail in the Discussion section.

Figure 3.2 Basic biochemical components' spectra used in the fitting of Raman spectra measured from K562 cells. (a) Actin; (b) Albumin; (c) Triolein; (d) Phosphatidylcholine; (e) DNA; (f) RNA; (g) Glycogen

The Raman spectra of live, apoptotic, and necrotic K562 cells were shown in Fig. 3.3. The spectrum for each group was obtained by averaging over 20 different cells and all spectra were offset accordingly on the y dimension for the clarity purpose. The standard deviations of the spectra were calculated and were superimposed on the averaged spectra as shown by the grey dotted line around curve (a), (b) and (c). The standard deviations of the spectra are small for most wavenumbers in the spectral range of interest except in the region around 1650 cm^{-1} , which is assigned to amide I bonding of proteins. This observation suggests that the inter-cell variation was low in cells of the same type due to the well regulation of contents in the cells. The large standard deviation at the protein band 1650 cm^{-1} may be due to the possibility that K562 cells were not synchronized in the cell cycle thus demonstrated various levels of protein expression in the different stages of the cycle.

Figure 3.3 Averaged Raman spectra of twenty (a) live K562 cells, (b) apoptotic cells and (c) necrotic cells. The standard deviations of the spectra were superimposed on the averaged spectra as shown by the grey dotted lines, which are too small to observe at most wavenumbers. Curve (d) was the difference between spectra (a) and (b) while curve (e) was the difference between spectra (a) and (c).

By visual inspection, it can be noted in Fig. 3.3 that the Raman peak at 734 cm^{-1} increased in apoptotic cells and decreased in necrotic cells compared to normal cells. This band was assigned to choline groups of phospholipids and it indicated the increase of membranous lipids in apoptotic cells and the decrease of membranous lipids in necrotic cells.

Raman peaks at 794 cm^{-1} , 1098 cm^{-1} and 1356 cm^{-1} were assigned to O-P-O, PO^{2-} nucleic acids backbone vibration and DNA-purine bases bonds of polynucleotide chain. From the basic components spectra, the peak around 1592 cm^{-1} is solely associated with nucleic acids. These bands were consistently lower in necrotic cells compared to normal cells indicating a significant reduction in both DNA and RNA concentration in necrotic cells, whereas the DNA/RNA related peak reduction in apoptotic cells was observed only at peak 794 cm^{-1} and the region around 1098 cm^{-1} .

Proteins have a prominent peak at 1011 cm^{-1} that was assigned to the symmetric ring breathing mode of phenylalanine¹⁰⁹ and does not overlap with Raman peaks of other components. This peak intensity notably decreased in necrotic cells but no significant change in intensity was observed in apoptotic cells. Raman peak at 1462 cm^{-1} can be assigned to CH_2 bending mode found primarily in proteins and lipids. The intensity at this wavenumber decreased in the necrotic cell spectra whereas an increase in this peak was observed in the apoptotic cell spectra compared to live cells. At the region of 1672 cm^{-1} , there was a significant increase in intensity for apoptotic cells while on the other hand there was a relatively large drop in intensity for necrotic cells. This region is assigned to $\text{C}=\text{O}$ stretching mode of proteins and also $\text{C}=\text{C}$ lipids stretch⁸⁰. The Raman peaks of proteins and lipids overlap significantly at 1462 cm^{-1} and 1672 cm^{-1} , so the changes in these regions do not directly indicate the alteration in protein or lipid level.

Fig. 3.4 shows the average Raman spectra of live, apoptotic and necrotic K562 cells and the fittings to the combination of basic biochemical component spectra as in Fig. 3.2. The Raman spectrum of empty sample well was also included in the fitting to improve the goodness of fitting. Each fitting coefficient was divided by the sum of all coefficients (excluding the coefficient for the spectrum of empty sample well) and converted to a percentage, to represent the relative contributions of basic biochemical component spectra to the bulk cell spectra. For example, 61% of the live cell spectrum was contributed by protein, 7.4% by triolein, 16.7% by phosphatidylcholine, 5.1% by DNA, 7.2% by RNA and 2.7% by glycogen according to Fig. 3.4(a). The contributions of protein, DNA and glycogen were lower, whereas lipids and RNA contents were higher in apoptotic cells as compared to live cells. In contrast, the protein content in

necrotic cells was higher than live and apoptotic cells while lipids, nucleic acids and glycogen content were lower as compared to others.

Figure 3.4 Mean Raman spectra of (a) live, (b) apoptotic and (c) necrotic cells and the corresponding fittings. The thick solid lines represent the measured spectra of cells while the thin dotted lines represent the fitting. The percentages to the right of each plot represent the fitting coefficients of the basic components that have been divided by the sum of all coefficients to represent the relative contributions of basic biochemical component spectra to the bulk cell spectra.

Fig. 3.5 shows the fitting coefficients for each basic biochemical component in live, apoptotic and necrotic cells. The error bars indicate the standard deviations of the fitting coefficients measured in twenty different cells. The star symbol (*) above the

error bars indicates that the relative amount of the specific biochemical component is statistically different between two groups at a significance level of $p < 0.05$. The amounts of triolein, phosphatidylcholine and RNA were significantly higher while DNA content was lower in apoptotic cells than in live cells. However, no significant differences were observed in the levels of protein and glycogen in apoptotic cells as compared to the control group (live cells). Moreover, all of the biochemical components in necrotic cells were significantly lower than live cells. In this study, we performed a direct comparison in the amounts of each biochemical component between apoptotic and necrotic cells. The analysis in Fig. 3.5 indicates that the amounts of all the biochemical components in necrotic cells were lower than that in apoptotic cells. A Wilcoxon signed-rank test result shows that the differences in all components between these two death modes are significant to a level of $p < 0.05$.

Figure 3.5 Fitting coefficients of each basic biochemical component in live, apoptotic and necrotic cells. (*) indicates a significance level of $p < 0.05$ obtained by Wilcoxon signed-rank test. “Phos” represents “Phosphatidylcholine”.

Figure 3.6(a) 2-D and (b) 3-D PCA plots show the separation of data based on different modes of cell death. The percent variance captured by each PC is shown in parenthesis along each axis in (b).

PCA was performed on the raw cell spectra and the scores for the first ten principal components were analyzed with Kruskal-Wallis one-way analysis of variance. It was found that the means of PC 2 and PC 3 scores show significant differences among the three groups of cells. Although PC 1 accounts for the most variance in the data sets, it does not show significant difference across the groups in Kruskal-Wallis analysis. Two-dimensional plots were constructed with different combination of scores for the first three principal components (PC1, PC2 and PC3) and a three-dimensional plot was also constructed with the three sets of scores as shown in Fig. 3.6. From Fig. 3.6(a), it was found that necrotic cells can be easily distinguished from live and apoptotic cells by the first pair of principal components. The main discriminant was the score of PC 2, where necrotic cells have negative scores while live and apoptotic cells have positive

scores. The PC 1 score distribution of apoptotic cells was narrower than that of live and necrotic cells where all cells score positively in the first principal component. Live and apoptotic K562 cells were not separable with PC 1 and PC 2 but they could be separated by the scores of PC 3, which were negative for live cells and positive for apoptotic cells. However, necrotic cells have a scattered distribution over the range of PC 3 scores for both live and apoptotic cells and thus there is not any pair in the first three principal components that could be used to separate three groups of cells effectively. A three dimensional PCA plots employing all the first three PCs was then constructed as in Fig. 3.6(b) and showed excellent separation of apoptotic and necrotic cells with live cells in between the two groups.

3.6 Discussion

3.6.1 Selection of Basic Biochemical Components

Seven basic biochemical components were selected because of the following reasons. Actin and albumin were used to represent proteins. Two types of protein were chosen to account for the varieties and complexities of cellular proteins. By visual inspection of their Raman spectra, they are almost identical and show significant overlapping in most of the major peaks. To quantify their similarity, we treated their spectra as vectors in the wavenumber space and calculated the dot product of their normalized spectra. The dot product indicates the degree of similarity between spectra, which ranges from 0 to 1. A larger dot product suggests a higher similarity. The similarity between actin and albumin is 0.91. The introduction of the second protein into this study is to improve the performance of LSR method as the addition of albumin spectra has significantly reduced the fitting residuals during spectral

decomposition. Thus in this chapter, the relative contribution of actin and albumin are summed and regarded only as protein.

Phosphatidylcholine and triolein were chosen to represent lipids in cells. The presence of a 734 cm^{-1} Raman band in the spectrum of phosphatidylcholine corresponds to choline group of cell-membrane phospholipids⁸². Thus, the amount of phosphatidylcholine from the fitting will be useful to indicate the amount of membranous lipids in cells and triolein will give indication of non-membranous lipids.

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are two main types of nucleic acids found in all living organisms. Both of them were polynucleotides, where each of their subunit was made up of a pentose sugar, a phosphate group and a nitrogenous base. In spite of the close chemical similarity, they were highly specific in carrying out their own function where DNA stores genetic information and RNA is vital in protein synthesis to translate genetic information from DNA base sequences into functional sequences of amino acids. Thus, the amount of DNA gives indication of the nuclear content and amount of RNA gives indication of the protein synthesizing activity in cells.

Glycogen is the main form of polysaccharides in cells. Glycogen functions mainly as energy storage in cells, and it is the secondary long term cellular energy storage form after lipids. The amount of glycogen in cells is relatively low compared to lipids because it is less compact and has lower energy storage efficiency. It is found mainly in active cells which require rapid generation of energy such as liver cells and muscle cells. However, even in muscle cells, glycogen is found in low concentration (about 1% to 2% of muscle mass). Thus, the amount of glycogen is expected to be small in K562 cells in our study.

Besides these four classes of organic molecules, the combination between them forms conjugates or complexes that may contribute to the cell spectra as well. The examples of these conjugates include lipoproteins, glycoproteins, and riboproteins. Inorganic molecules are also found in living cells such as calcium, iron, zinc, and some of them can bind to proteins as cofactor compounds. This small amount of molecular constituents may contribute to the residual of our fitting because they are not accounted for by the basic biochemical components selected in this study.

3.6.2 Biochemical Component Analysis Revealing Biochemical Changes Underlying Apoptosis and Necrosis

A. Biochemical changes in apoptotic cells

It can be seen in Fig. 3.5 that the level of protein in apoptotic cells was not significantly different from live cells. It is known that the initiation of caspase cascade reaction during apoptosis activates effector caspase proteins that cleave other protein substrates within the cell, for example, breaking down cytoskeletons^{110, 111}. Consequently, the increase in caspase protein is likely counteracted by the breakdown of other cellular proteins, which would result in a minute change in the total protein amount in cell^{112, 113}.

The levels of triolein and phosphatidylcholine in apoptotic cells showed significant increases relative to the control group in Fig. 3.5, which can be explained by their roles in apoptosis. Triolein, a non-membranous lipid, increases in apoptotic cells due to the accumulation of unsaturated lipids in cytoplasm forming lipids bodies. This finding agrees with a previous study⁸², where Zoladek et al. took Raman images in live and apoptotic human breast cancer cells using 1659 cm⁻¹ band to visualize lipids distribution over 6 hours. In contrast, the increase in phosphatidylcholine indicates the

accumulation of membranous lipids in cells mainly attributed to the formation of apoptotic bodies. The packaging of organelles and cellular contents into apoptotic bodies and the event of membrane blebbing in apoptosis require the synthesis of new membrane lipids¹¹⁴.

Decreased DNA content in apoptotic cells relative to live cells in Fig 3.5 suggests that most cells in this study were in the late apoptosis stage. In the early apoptosis stage, nuclear condensation occurs where chromatin is compacted against perinuclear envelope in the hallmark process of apoptosis called pyknosis and DNA content is increased as confirmed in a previous Raman study⁸². However, nuclear condensation in apoptosis is a short process. In the fragmentation process after condensation, DNA is cleaved into short fragments to be packed in apoptotic bodies, which causes reduction in the intensity of DNA peaks mainly at O-P-O phosphodiester backbones band at 794 cm^{-1} . The breakdown of phosphodiester bonds and DNA bases during nuclear fragmentation is the hallmark event of late apoptosis. In addition, cytosine arabinoside used in this study to induce apoptosis in K562 cells will induce erythroid differentiation in K562 cells where they progressively lose their DNA content even before apoptosis cascade is activated. This expected drop in DNA content is confirmed by the observed decrease in DNA content based on the fitting result in Fig. 3.5. RNA shows a slight increase in apoptotic cells compared to live K562 cells. This can be attributed to the increase in RNA that directs the synthesis of caspase proteins¹¹⁵.

Even though apoptosis is an active process that requires energy, no significant reduction in the relative amount of glycogen in apoptotic cells is observed. It may suggest that glucose supply in the culture medium used for culturing these apoptotic cells is sufficient in this process. Since glucose is favored as the primary and

immediate source of energy in apoptosis, there is no need to initiate glycogenolysis to break down glycogen for energy if the glucose supply is adequate.

B. Biochemical changes in necrotic cells

In necrotic cells, the relative amounts of all the basic biochemical components showed significant reduction compared to the control group. The reduction in DNA content is due to DNA degradation by the breakdown of both phosphodiester bonds and DNA bases. Different from apoptosis where DNA is first broken down into nuclear fragments, DNA degradation in necrosis is a random event and nuclear fragments are not packed into vesicles to facilitate the uptake and incorporation into neighboring cells. RNA level in the cells reduces in necrotic cells because necrosis is a passive mechanism of cell death that requires no new protein needs to be synthesized. The decrease in the amount of phosphatidylcholine that represents membranous lipids suggests the loss of membrane integrity in necrotic cells. The rupture of cell plasma membrane causes the leakage of cell content into the surroundings as the ruptured membrane allows the random diffusion of cell content across the membrane. This explains the decrease in cellular lipids, proteins content and glycogen level in necrotic cells even though necrosis does not require energy.

3.6.3 Comparison of PCA and BCA in Cell Death Classification

We have explored the use of biochemical component analysis (BCA) and principal component analysis (PCA) in analyzing Raman spectra of different groups of live and dead cells. Previous studies have demonstrated that either method is capable in highlighting the variance in Raman spectra across different groups of cells^{80, 83, 116}; however, little effort has been made to compare the classification performance and interpretability of the two methods. Hence, we have fed the features extracted from

both methods, i.e. the fitting coefficients of each biochemical coefficient in BCA and the scores of principal components in PCA, into a linear discriminant classifier and a leave-one-out cross validation method was employed to train the classifier. First, we compared the resulting classification accuracies of PCA and BCA by using a single feature extracted from each method. When the score of a single principal component was used, the classification accuracy is 68.3% for PC 1, 91.7% for PC 2, 63.3% for PC 3, 60.0% for PC 4 and 63.3% for PC5. The classification accuracy using the fitting coefficient of a single basic biochemical component is 70.0% for protein, 83.3% for both triolein and phosphatidylcholine, 80.0% for DNA, 73.3% for RNA and 66.7% for glycogen.

Figure 3.7 The spectra of first three principal components in PCA, where (a) is PC 1, (b) PC 2 and (c) PC 3.

Fig. 3.7(a) shows that PC 1 resembles the average cell spectrum. It is noted that the classification with PC 1 score is 68.3% which is lower than the classification accuracy of PC 2 even though PC 1 accounts for most of the variance in cell spectra. By decomposing PC 1 with regards to the basic biochemical components, i.e. performing BCA on PC 1, we find that the majority of PC 1 is contributed by protein (58%) and phosphatidylcholine (20%) while other biochemical components contribute less than 10% each to PC 1. Since there is no significant change in the relative amount of protein in live and apoptotic cells as shown in Fig. 3.5 and PC 1 is largely contributed by protein spectrum, the low classification accuracy using PC 1 could be attributed to its high protein content. Decomposing PC 2 spectrum with regards to the basic biochemical components returns a large fitting residual (result not shown). This is likely due to the fact that PC 2 is orthogonal to PC 1 in the vector space thus does not show strong correlation to the basic biochemical components spectra to which PC 1 can be fit well. However, it is noted that PC 2 as in Fig. 3.7(b) captures two prominent Raman peaks at 1098 cm^{-1} which corresponds to O-P-O DNA backbone vibration and 1672 cm^{-1} which corresponds to the C=O stretching mode of proteins and C=C lipids stretch. This observation explains why PC 2 can be used to classify live and dead cells with good accuracies, considering that both lipids and DNA demonstrate significance differences between live and dead cells as shown in Fig. 3.5. Similar to PC 2, the fitting of PC 3 using BCA also returns a large fitting residual. PC 3 as in Fig. 3.7(c) captures three peaks, respectively, at 734 cm^{-1} which corresponds to choline group of phospholipids, 1011 cm^{-1} which can be assigned to the symmetric ring breathing mode of phenylalanine and 1462 cm^{-1} which corresponds to the CH₂ bending mode of proteins and lipids. This result suggests that the combination of proteins' and lipids'

peaks is not a good parameter in distinguishing cell death modes as the classification accuracy using PC 3 is only 63.3%.

When using the fitting coefficient of BCA to classify live, apoptotic and necrotic cells, both lipids (triolein and phosphatidylcholine) score the highest accuracies among all basic biochemical components with 83.3%, followed by DNA which scores 80%. Proteins and glycogen have lowest classification accuracies, which are 70% and 66.7% respectively. This can be due to the fact that the fitting coefficients for both proteins and glycogen show no significant differences among cell groups in Wilcoxon signed-rank test as shown in Fig. 3.5.

Table 3.2 Classification accuracies using two principal component scores obtained from PCA

	PC 1	PC 2	PC 3	PC 4	PC 5
PC 1	68.3%	95.0%	75%	85.0%	78.3%
PC 2		91.7%	96.7%	95.0%	95.0%
PC 3			63.3%	80.0%	80.0%
PC 4				60.0%	78.3%
PC 5					63.3%

Table 3.3 Classification accuracies using two fitting coefficients obtained from BCA

	Protein	Triolein	Phos	DNA	RNA	Glycogen
Protein	70.0%	86.7%	93.3%	90.0%	90.0%	86.7%
Triolein		83.3%	93.3%	96.7%	90.0%	86.7%
Phos			83.3%	86.7%	83.3%	76.7%
DNA				76.7%	90.0%	90.0%
RNA					73.3%	80.0%
Glycogen						66.7%

To explore the minimum number of components for high classification accuracy, the use of two PC scores or fitting coefficients in conducting LDA analysis has been tried and the classification results are presented in Tables 3.2 and 3.3. Generally, when two components from PCA or BCA are used, the classification accuracies will be improved. For example, when the score of PC 1 alone is included in the classifier, the accuracy is 68.3%. In contrast, when PC 2 score is also incorporated into the classification, the accuracy will be improved to 95%. Similarly, the accuracy of using only PC 3 score will be improved from 63.3% to 75% when the score of PC 1 is included in the classifier. The highest accuracy achieved is 96.7% when scores of PC 2 and PC 3 are used.

The classification accuracies for BCA also improved when two fitting coefficients are used. For example, the classification accuracy by using the fitting coefficient of proteins is improved from 70% to 86.7% when the fitting coefficient of triolein is added, while the addition of fitting coefficient of phosphatidylcholine can improve the classification accuracy to 93.3%. The highest classification accuracy that can be achieved is 96.7% when the fitting coefficients of DNA and triolein are used. This result again indicates that the combination of DNA and membranous lipids can be used to discriminate between live and dead cells with excellent accuracies. This combination has been explored recently by Zoladek et. al.¹⁰⁴ for Raman imaging of apoptosis in human breast cancer cells. Besides DNA and lipids, several studies^{80, 116, 117} has also highlighted the changes in protein amount between live and dead cells. For PCA method, a combination of scores from the first three principal components results in a classification accuracy of 98.3%. The addition of another score from the fourth PC can further improve the classification accuracy to 100%. In BCA, the combination of

three fitting coefficients for proteins, DNA and lipids yields a classification accuracy of 100% in this study.

It is worth noting that the highest classification accuracy achieved using the fitting coefficients from BCA method is slightly better than that achieved with the scores of principal components from PCA method. However, our results have also shown that the classification of live, apoptotic and necrotic cells based on both PCA and BCA had excellent accuracies, and neither method has shown a clear superiority over the other method in this respect. However, principal component analysis has a disadvantage in that it does not reveal the biological basis of the changes observed in the Raman spectra. Most principal components (except PC 1) extracted in PCA do not carry physically meaningful information about biochemical changes in cells. The BCA method, on the other hand, is capable of extracting the relative concentrations of biochemical components contributing to the spectra that could provide useful information about the physiological and structural changes in different cell death modes. Proper utility of the BCA method however requires good knowledge of the underlying biochemical constituents in a cell in order to achieve good fitting result with minimal residual. Thus, PCA analysis method can be favorable when the spectra of basic constituents of samples are unavailable.

3.7 Conclusion

In this study, we exploited the potential of Raman spectroscopy by decomposing the full Raman spectrum with in-house Biochemical Component Analysis method to quantify the changes in cellular constituents in each death mode. Cell spectra were decomposed into the contributions from several basic biochemical components including protein, lipid, nucleic acid and glycogen by employing a least

square regression algorithm. The fitting results of apoptotic cells show significant increases in lipid and RNA and a decrease in the DNA content as compared to live cells. In contrast, significant reductions in the levels of all basic biochemical components are observed in necrotic cells compared to both normal and apoptotic cells. Then, cell death classification accuracies based on features extracted from PCA and LSR methods are compared using LDA. Both methods showed excellent performance in classifying live, apoptotic and necrotic cells and neither of the analysis method shows obvious superiority over the other in this respect. Having similar cell death modes classification performance with BCA-PCA method, BCA-LSR method could provide insight into the biological basis changes in different cell groups.

Besides demonstrating the ability of BCA method in elucidating the biochemical compositions in different types of cell, we also demonstrated a simple Raman spectroscopic technique to measure and analyze the different death modes of individual leukemic cells in suspension. In addition to qualitatively study the intensity changes of some prominent peaks in cell Raman spectra; the detection of fine biochemical changes in the cell spectra will help develop Raman spectroscopy techniques to monitor cellular changes in various biomedical researches as well as potential medical diagnosis tool for various diseases in the tissue level.

Chapter 4 Investigation of Depth Profile of Optical Signals Measured by the Cone and Cone Shell Configurations in Layered Tissue Phantoms

4.1 Introduction

Depth sensitive optical measurements can be achieved in two ways, the contact fiber optic based setups and the non contact lens-based setups. A major disadvantage of fiber optic based setups is the inconsistent pressure exerted on biological tissues which can cause spectral distortion that severely affects the diagnostic accuracy of this technique. Besides that, the contact between the probe and the patient's skin especially on infectious sites may cause probe contamination and elevate the risk of disease transmission across patients. Alternative non-contact measurement methods are desirable as alternative to fiber optic based setups to overcome these complications. Therefore, in this report, we will be focusing on the non contact lens-based setups.

Generally, lens-based setups use the combination of lenses to achieve the excitation and collection volumes that would form cones in an optically transparent medium. One weakness of this setup is the limited sensitivity to optical signals originating from subsurface layers due to the contribution from shallower layers in a turbid layered medium such as human skin. A common optical instrument which employs the cone geometry in the illumination and detection configurations is the microscope. To improve the depth sensitivity of current lens-based setup, we introduced a novel cone shell illumination geometry in place of the conventional cone geometry. In a cone shell illumination volume, the volume of shallower layers being excited is effectively reduced compared to a cone illumination configuration. The measured signal will contain lesser signal from the overlaying layers, thus improving the depth sensitivity of the system.

In this study, we conducted a series of Raman experiments to obtain the performance of depth sensitive measurements using a microscope objective lens, in which the focal depth is varied to cover the layers of interest, in transparent and turbid tissue phantoms. Then, the phantom experiment is repeated using the novel cone shell configuration in turbid tissue phantom and the depth sensitivities achieved by both cone and cone shell configuration are compared.

4.2 Refractive Index Mismatch at the Probing Interface

Refraction due to refractive index mismatch at the probing interface may significantly affect the depth resolution of Raman microscopy using the microscope objective lens. Everall¹²⁵ has performed an analysis based on ray tracing and demonstrated that the shape and volume of the laser focus will be distorted if the beam is focused into a material with a different refractive index. The position and depth of laser focus will increase dramatically when the laser beam is focused deeper into the sample. This refractive aberration can be ameliorated by using an immersion objective¹²⁵ but this optical configuration will not be suitable for a handheld Raman probe. Delhaye *et al.*¹²⁶ has derived an expression to estimate the maximum depth of focus, given the sample refractive index, the numerical aperture and the degree of filling of objective aperture (ratio of beam radius, r_b , to aperture radius, r_a) are known. Thus, in our case, the changes in the depth of focus due to refractive index mismatch between air and skin can be easily calculated as all the parameters are known.

Figure 4. 1 Schematic diagram showing the effect of refractive index mismatch between the skin and air in (a) normal microscope lens and (b) the proposed light collection system.

The refraction effect on depth resolution using a common microscope objective lens is illustrated in Fig. 4.1 (a). We can see that the depth of focus is located at a distance, z_o , beneath the sample surface when the refractive indices of air and skin are identical ($n_1=n_2$). However, in the actual case, the refractive index of skin is higher than air, with 1.34 for epidermis and 1.41 for dermis¹²⁷. Thus, the maximum true depth of focus will be located at a much deeper position at distance, z_t , underneath the skin surface. The true depth of laser focus in skin tissue can be estimated by applying the following equation:

$$z_t = z_o \left[m^2 \cdot \frac{NA^2(n^2-1)}{(1-NA^2)} + n^2 \right]^{0.5} \quad (4-1)$$

where,

m = degree of filling of objective aperture, i.e. the radial location of the light ray on the lens plane relative to the full radius of the lens;

NA = numerical aperture of objective lens;

$n = n_1 / n_2$

Since the light passing through the objective lens is converging, the volume of the true focal point under the refractive aberrations will be increased and the shape will become elongated in z-axis and this will severely affect the depth resolution of Raman measurement.

By using a cone shell illumination configuration, the changes in focal volume by refractive aberrations can be minimized as shown in Fig. 4.1 (b). We can see that the maximum true depth of focus is equal to that in configuration (a) but the focal volume is comparatively smaller than (a) and can be manipulated by changing the width of the laser ring. This advantage may contribute to the improvement in depth sensitivity in performing layer specific Raman measurements.

4.3 Depth Profile of Cone Configuration in Transparent and Turbid Media

4.3.1 Experimental Setup

A micro-Raman spectrometer system (inVia, Renishaw, UK) coupled to an upright microscope (Alpha 300, WiTec, Germany) in the backscattering configuration was used for Raman measurements in the phantom experiment. A 633 nm He-Ne laser with a power of 20 mW on the sample was used for the excitation illumination time was 10 seconds.

Figure 4. 2 Schematic diagram of a two-layer tissue phantom. Rhodamine 6G and Urea were added into the top and bottom layer at concentrations of 1 mM and 2M respectively. d

represents the thickness of the top layer. Four different thicknesses of top layer were investigated, i.e. 180 μm , 360 μm , 540 μm and 720 μm .

Skin tissue phantoms mimic the layered structure of the skin with controllable layer thicknesses and optical properties. Agar phantoms are cheap and easy to fabricate thus good for testing a new technique. In total two sets of agar phantoms were fabricated as shown in Figure 4.2, one with separate elastic scattering components and the other without such components. The first set of two-layered agar phantoms was created using two distinct Raman scatterers, one in each layer. Urea and Rhodamine 6G are good candidates for the Raman scatterers since they exhibit strong Raman signals under 633-nm excitation and they have prominent peaks that do not overlap with each other, the former at 1002 cm^{-1} and the latter at 1362 cm^{-1} . Rhodamine 6G at a concentration of 1 mM will be added to the top layer while urea at a concentration of 2M will be used in the bottom layer. In the first set of phantoms, four different thicknesses of the top layer were prepared at 180 μm , 360 μm , 540 μm and 720 μm and the thickness of the bottom layer was 10 mm to be optically semi-infinite in order to investigate the change in Raman signals from both layers when varying the depth of laser focus. The peak intensities at 1002 cm^{-1} and 1362 cm^{-1} were plotted against the depth of laser focus.

The second set of agar phantoms was created with added elastic scatterers to investigate the efficacy of Raman depth selective measurement in a turbid media using conventional Raman microscopy in backscattering configuration. Intralipid 20%^{118, 119} was used as the scatterer. Intralipid, a fat emulsion similar to milk, has been used widely in optical experiments to simulate the elastic scattering properties in biological tissues¹²⁰. The reduced scattering coefficient of Intralipid 20% was reported to be 260 cm^{-1} at the excitation wavelength of 633 nm¹⁴⁷. To match the reduced scattering

coefficients of epidermis and dermis at the excitation wavelength of 633 nm, which are reported to be 55 cm^{-1} and 35 cm^{-1} respectively¹⁴⁶, intralipid was added into the top layer with a dilution ratio of 1:4.7 and into the bottom layer at a dilution ratio of 1:7.4. Such dilution procedure assumed that μ'_s are proportional to the concentration of intralipid. The tissue phantoms with separate elastic scatterers were tested with the same experimental setup as the first phantom study, and the results were compared to investigate the effect of light scattering on the Raman depth profile from both layers in a skin tissue model.

4.3.2 Results and Discussion

A. Phantoms without separate elastic scatterers

It should be pointed out that even there were no separate absorbers and elastic scatterers in this set of phantoms, the absorption coefficient of the phantoms due to Rhodamine and Urea, and the scattering coefficient due to agarose, may not be totally negligible¹²¹. The depths of measurement of the results in this section were corrected using equation 4-1 to account for the refractive index mismatch at the air-phantom interface. The effect of refractive index mismatch on the measurement focal depth will be discussed in the following section.

Figure 4.3 Raman peak intensities at 1362 cm^{-1} (Rhodamine) plotted against the depth of focus in tissue phantoms with various thicknesses of the top layer (A: $180\text{ }\mu\text{m}$; B: $360\text{ }\mu\text{m}$; C: $540\text{ }\mu\text{m}$; D: $720\text{ }\mu\text{m}$).

Figure 4.4 Raman peak intensities at 1002 cm^{-1} (urea) plotted against the depth of focus in tissue phantoms with various thicknesses of the top layer (A: $180\text{ }\mu\text{m}$; B: $360\text{ }\mu\text{m}$; C: $540\text{ }\mu\text{m}$; D: $720\text{ }\mu\text{m}$).

Fig. 4.3 and 4.4 show the Raman intensity at 1362 cm^{-1} (Rhodamine) and 1002 cm^{-1} (urea) versus the depth of measurement beneath the phantom surface. In Fig. 4.3, we can see that the highest intensity for A is achieved when the focus depth is around $100\text{ }\mu\text{m}$ while B, C and D achieved highest intensity at focus depth around $250\text{ }\mu\text{m}$.

Decreases in the intensities are observed when the depth of measurement is increased within the thickness of the top layer. The observation can be attributed to the attenuation of signals from deeper layers that travelled through a larger distance. The effect of attenuation at small focal depth is less significant as the peak intensities for all samples are similar where the focal depth is smaller or equal to 200 μm . The intensities reduced to half of their peak values as the depth of focus increases close to the thickness of top layer. This observation shows that the half maximum of the peak value could be indicative in the estimation of thickness of a thin sample layer. From Fig 4.3, we can also see that significant contribution from the top layer is still observable when the focal spot is moved down to the bottom layer.

Fig. 4.4 shows the signals collected from bottom layer. We can see that the peak intensities of the curves are always slightly greater than the depth of the bottom layer from the sample surface. For example, the highest intensity for sample A is achieved when the depth of focus is increased to 200 μm . Similarly, sample B has the highest intensity at 1002 cm^{-1} close to its depth at around 450 μm , C at 650 μm and D at 800 μm . Different from Fig. 4.3, the peak intensities decrease from sample A to D. This observation shows that Raman signals from deeper a layer experienced greater attenuation compared to shallower layers due to light diffusion and the increase in light path.

In this phantom study, a point focus illumination and signal collection configuration is adopted by using Raman microspectroscope. A clear boundary between the top and bottom layer of the phantom is expected to be reflected on the result obtained. This would mean that most signals collected when the focus spot falls in the top layer are contributed by Rhodamine while most signals collected are contributed by urea when the focus spot falls into the bottom layer. In an ideal

refractive index match case, the excitation light passing through a microscope lens will converge to form a point focus in the phantom. However, in the actual case as in this study, there is a mismatch between the refractive indices of air and phantoms, causing the laser that passes through the sample surface to be refracted to a greater depth and form a line focus that is elongated in the direction parallel to the light entering the microscope lens. The formation of a line focus can cause out-of-focus Raman signals from both shallower and deeper layers to be collected. This can be observed in Fig. 4.3 where a significant contribution from top layer is still observable when the depth of focus spot fell within the bottom layer. Whereas, in Fig. 4.4, Raman signal from urea can be observed before the focus spot is moved into the bottom layer. This will reduce the depth resolution of Raman microspectroscopy and a method to minimize this problem due to refractive index mismatch at the interface will be discussed in the following section.

B. Phantoms with separate elastic scatterers

In this set of phantoms, intralipid was added into both layers as separate elastic scatterers to investigate the effect of scattering on the depth profile of Raman signal.

Figure 4.5 Raman peak intensities at 1362 cm^{-1} (Rhodamine) plotted against the depth of focus in tissue phantoms with various thickness of top layer (A: $180\text{ }\mu\text{m}$; B: $360\text{ }\mu\text{m}$; C: $540\text{ }\mu\text{m}$; D: $720\text{ }\mu\text{m}$). Intralipid was added into the phantoms to achieve a scattering coefficient of 100 cm^{-1} at 633 nm .

Figure 4.6 Raman peak intensities at 1002 cm^{-1} (urea) plotted against the depth of focus in tissue phantoms with various thickness of top layer (A: $180\text{ }\mu\text{m}$; B: $360\text{ }\mu\text{m}$; C: $540\text{ }\mu\text{m}$; D: $720\text{ }\mu\text{m}$). Intralipid was added into the phantoms to achieve a scattering coefficient of 100 cm^{-1} at 633 nm .

In Fig. 4.5, we can see that the highest intensity for A is achieved when the focus depth is around 50 μm while B, C and D achieved highest intensity at focus depth, around 180 μm . In the first set of phantoms in which there were no separate elastic scatterers, the focal depth was 100 μm for A and 250 μm for the rest. This difference may be due to the addition of intralipid that increases the scattering coefficient of the phantoms. Excitation light may be scattered more significantly and formed a larger focus spot as compared to the case without separate elastic scatterers. A larger volume of the sample will be illuminated and signal from planes both above and below the desired layer could have contributed to the overall Raman scattering. In this case, a larger volume of Rhodamine layer will be illuminated and generate higher Raman signal than the zero scattering case upon a small increment in the depth of focus near the sample surface.

Fig. 4.6 shows the signals collected from bottom layer. A similar trend was observed as compared to the first set of phantoms as the peak intensity of bottom layer is always deeper than its actual depth from sample surface. For example, the highest intensity for sample A is achieved when the depth of focus is increased to 250 μm . Similarly, sample B has the highest intensity at 1002 cm^{-1} close to its depth at around 400 μm , C at 650 μm and D at 800 μm . The decrease in peak intensities from sample A to D is also observed as in the first set of phantoms. This observation is due to the attenuation of Raman signals originating from a deep layer when travelling to the surface of the sample. The results in Fig. 4.3 and 4.5 show that the longer the distance travelled by Raman scatterings, the greater is the light attenuation, which causes significant loss in the intensities of the collected signals. Hence, a reliable method to recover subsurface Raman signal is vital because the attenuation of Raman signal from subsurface skin lesions is expected to be more profound as the thickness of lesions can

be ranged from a few micrometers to 2 millimeters at the basal layer of the epidermis¹²².

Figure 4.7 Ratio of peak intensities at 1002cm^{-1} to the total intensities of peaks at 1002 cm^{-1} (Urea) and 1362 cm^{-1} (Rhodamine) plotted against depth of measurement with various thickness of top layer. (a) with elastic scatterers and (b) without elastic scatterers.

In Fig. 4.7, the sensitivity of Raman microspectroscopic configuration to the subsurface layer is shown by taking the ratio of peak intensities at 1002 cm^{-1} to the total intensities of peaks at 1002 cm^{-1} (Urea) and 1362 cm^{-1} (Rhodamine). In case (a), these ratios are close to 0.2 at the surface of the sample and gradually increases, as the depth of measurement increases, until reaching the peaks (>0.9) around $200\text{ }\mu\text{m}$, $400\text{ }\mu\text{m}$, $500\text{ }\mu\text{m}$, and $700\text{ }\mu\text{m}$ respectively, which are close to their actual depths. In case (b), as the thickness of the top layer increases, the sensitivity to urea at bottom layer was low at the shallower depth of measurement. For example the ratio is around 0.2 for a depth of measurement up to $400\text{ }\mu\text{m}$ in the third curve and $600\text{ }\mu\text{m}$ in the fourth curve. Different from case (a), a relatively sharper increase in the ratio were observed in case without elastic scatterers before the ratios reach the peaks at depth of measurement close to the actual depth of bottom layer. In both cases, with and without separate elastic scatterers, the curves reach a plateau when the ratios reach above 0.9

as the depth of measurement increases. The further increase of focus depth below the boundary of two layers will not increase the sensitivity of this technique to the bottom layer. These results suggest that as the scattering property of a medium increases, mixture of signals from larger volume especially from the overlaying layers will be collected by a cone configuration which reduces the depth sensitivity of the measurements. Thus, we proposed a novel cone shell configuration to improve the depth sensitivity of a Raman microscope in turbid medium and its performance will be compared to the depth sensitivity achieved by a conventional cone configuration.

4.4 Cone Shell Configuration for Depth Sensitive Measurements

Figure 4.8 Schematic diagrams showing (a) cone illumination configuration of conventional Raman system, (b) novel cone shell illumination by focusing a laser ring. Refractive index match is assumed at the probing interface and the phantom is assumed to be optically transparent.

Here, we proposed a novel Raman excitation and collection configuration to enhance the depth selectivity of Raman spectroscopy. A ring of laser beam is focused by a converging lens to create a cone shell illumination, as shown in Fig. 4.8 (b), with the tip as the focus point. In this case, a smaller volume of the shallower layer will be excited by the excitation laser as compared to the typical configuration using the full cone of the laser beam. Raman signal out of the incident light path that are collected

by the focusing lens will be blocked by using a aperture slider to reduce the Raman signal from layers that are off focused.

A phantom study will be performed to compare depth sensitivity achieved by these two configurations; i.e. cone and cone shell configurations. Two layered agar phantoms with distinct Raman scatterers in each layer will be created. Rhodamine was added to the top layer and urea was added to the bottom layer at concentrations similar to that in section 4.3.1. However, in this set of phantom, the thickness of the top layer was made to be 2.5 mm and the thickness of the bottom layer was made to be greater than 1 cm to represent a semi infinite medium.

Firstly, the sample will be measured using a conventional Raman microscopy setup. Raman measurements are taken from 0 mm, which is the surface of the phantom, to 4 mm in depth with an increment of 0.5 mm per measurement. The intensities of urea and Rhodamine at different at 1002 cm^{-1} and 1362 cm^{-1} will be plotted against the depth of focus, as shown in Fig 4.9. Then the experiment is repeated with the proposed optical configuration shown in Fig. 4.8 (b) by inserting an aperture slider behind the aperture of microscope objective to block the central region of the incident laser beam and the backscattering Raman signal. The peak intensities of urea and Rhodamine will be plotted against the depth of focus and the sensitivity to the bottom layer is expected to be higher for a greater focal depth. The sensitivity to the bottom layer was estimated by taking the ratio of Urea intensity to the sum of Rhodamine and Urea intensities, as shown in Fig. 4.10.

Figure 4.9 Raman peak intensities of Rhodamine at 1362 cm^{-1} (top layer) and Urea at 1002 cm^{-1} (bottom layer) measured using a (A) cone configuration and (B) cone shell configuration, plotted against the depth of focus in tissue phantoms.

In Fig. 4.9 (A), the peak intensity of the top layer rises from the surface and reaches to the maximum at around 1.5 mm before decreasing as the focal depth increases to 4 mm. The peak intensity of the bottom layer rises slowly and continuously from the surface to 4 mm focal depth. In Fig 4.9 (B) where a cone shell configuration was employed, the peak intensity from top layer rises from the surface and reach to a maximum at around 1 mm before decreasing as focal depth increases to 4 mm while the peak intensity of the bottom layer increases slowly and continuously from the surface before reaching to a plateau at 3.5 mm focal depth onwards. It can be seen that the peak intensity of the top layer in a cone shell configuration drops relatively faster than in a cone configuration after reaching its maximum at around the centre of the top layer. The dashed lines in Fig 4.9 represent the boundary of top and bottom layer in the agar phantoms. It is clear that, in Fig 4.9 (B), the contribution of top layer near the boundary is relatively lower than that in Fig 4.9 (A) which suggests that a cone shell configuration is able to reduce the signal contribution from shallower layers in a turbid medium to improve the depth sensitivity of the measurements.

Fig. 4.10 shows the sensitivity to the bottom layer achieved by both the cone and cone shell illumination configurations. The sensitivity to the bottom layer was computed by taking the ratio of signal originated from the bottom layer (peak intensity of Urea) to the sum of signal intensity from both the top and bottom layers (peak intensities of Rhodamine and Urea). It can be seen that, the sensitivity to bottom layer of both configurations show continuous increasing trend when the focal depths increase from 0 mm to 4 mm. However, the depth sensitivity achieved by cone shell configuration is superior to the cone configuration at almost all measurement points from 1 mm onwards to 4 mm which suggests an enhancement in the depth sensitivity of a cone shell configuration by the rejection of off-focus signal originating from the overlaying layers.

Figure 4.10 Sensitivity to the bottom layer achieved by a cone and cone shell configuration plotted against depth of measurement.

4.5 Progressive Estimation Method to Recover Raman Spectra from Individual Layers

In Section 4.3, we demonstrated the contribution of shallower layers when performing deep measurements in a scattering medium. To reduce this off-focus signal, we added an aperture slider in the light path to create a cone shell illumination pattern which minimises the excited volume of the overlaying layers as well as to block the undesired signals originated from the off-focus region as described in Section 4.4. In this section, we explore the feasibility of improving the depth sensitivity of optical measurements in scattering media by developing a progressive estimation method. In this approach, the Raman signal contribution from the top layers of different thicknesses when performing a deep measurement will be quantified and subsequently being subtracted away from the measured spectra to obtain depth specific Raman spectra of individual subsurface layers. This approach involves no modification to the optical configuration and can be applied to any commercial Raman microscope system to improve its depth sensitivity.

A series of phantom experiments were carefully designed and performed to test and verify our progressive estimation method. In the phantom study, the concentrations of Raman scatterers and optical properties are assumed to be homogeneous in each layer and the intensity of Raman signal from each Raman scatterer is assumed proportional to its concentration. The Raman spectrum measured from the first depth is assumed to be contributed by the first layer only. The Raman spectrum of the second depth will be contributed by the second layer and the attenuated signal from the first layer. Similarly, the Raman signal measured from the third depth will be contributed by the third layer and attenuated signals from all the shallower layers. The attenuation can be represented by a scaling factor which is related to the distance travelled by the Raman light. Assigning an index number from 1 to N at each depth, from the smallest (corresponding to the

shallowest layer) to the largest (corresponding to the deepest layer), this scenario can be mathematically illustrated as below:

$$\begin{aligned}
 CR_1 &= R_1 \\
 CR_2 &= R_2 + \alpha_1 CR_1 \\
 CR_3 &= R_3 + \alpha_2 CR_2 \\
 &\dots \\
 CR_N &= R_N + \alpha_{N-1} CR_{N-1}
 \end{aligned}$$

where,

CR_N = Raman spectrum measured when targeting on depth N

R_N = Raman contribution from layers at depth N only

α_{N-1} = Scaling factor accounting for the attenuation of Raman signals from shallower layers at depths 0 through N-1.

Generally the Raman spectrum measured from the N-th depth, is contributed by the N-th layer and the off-focus Raman signals from all the shallower layers. The Raman light originated from the shallower layers is accounted by multiplying a scaling coefficient to the Raman spectrum measured from the (N-1)th depth. The scaling coefficient is approximated experimentally by taking the ratio of Raman intensity measured from two depths. To obtain the values of α for a range of focal depths, a set of two-layered agar phantoms with different top layer thicknesses were prepared according to the recipe and procedure published in an earlier report¹²³. The phantom was made in a cylindrical plastic petri dish with a diameter of 30 mm, in which the thicknesses of top layers was made to be 180 μm , 360 μm , 540 μm and 720 μm while the bottom layer was made to be 10 mm to represent a semi-infinite medium. Urea was added into the top layer at a concentration of 1 M while no Raman scatterer was added into the bottom layer. When the laser spot is focused at the bottom layer, any Raman signal of urea which is detected will be contributed by the overlaying layer. By taking the ratio of urea peak intensity measured from two depths across the boundary of the phantom, the

contribution of Raman signal originated from the overlaying layer or the scaling factor can be estimated. By estimating the Raman signals contributed from the overlaying layers accurately, we can obtain layer specific Raman spectrum from a subsurface layer with only two measurements using any conventional Raman microscope. It should be noted that, even though this method is developed using a Raman model, it can also be applied to other optical measurements such as fluorescence or diffuse reflectance measurements to obtain layer specific optical signals. The estimation of scaling factor will be clearly explained in the next paragraph with the aid of Fig. 4.11.

Figure 4. 11 Schematic diagram of excitation and detection configuration targeting at (a) the top layer and; (b) the bottom layer of a two-layered turbid agar phantom. Black solid line represents excitation light and red dashed line represents Raman light.

Fig. 4.11 shows Raman measurements from two depths in a two-layered agar phantom. Urea was added into the top layer which was represented in purple colour while a plain agar bottom layer was represented in light grey colour. R_1 refers to Raman signal originated from the top layer only, which is the Raman signal of urea with a peak at 1000 cm^{-1} . R_2 refers to Raman signal originated from the bottom layer only, which is zero as no Raman scatterer was added into the bottom layer. In Fig. 4.11 (a), the laser was focused

into the top layer, therefore the Raman spectrum measured (CR_1) contains only signals from top layer (R_1). In Fig. 4.11 (b), the laser was focused in the bottom layer forming a cone of laser light with the core mainly illuminating the top layer while the tip located in the bottom layer. Thus the Raman spectrum measured from the second depth (CR_2) contains signals from the bottom layer (R_2) and a portion of the attenuated signals from the top layer ($\alpha_1 CR_1$). However, as no Raman scatterer was added into the bottom layer, $R_2 = 0$, the mathematical model of Raman signal measured from the second depth can be simplified to $CR_2 = \alpha_1 CR_1$. By taking the ratio of Raman signal measured from the bottom layer to those from the top layer (CR_2 / CR_1), the contribution of the Raman signal from the top layer or the scaling factor α_1 can be estimated.

In this study, we investigated the changes in the scaling factor with different top layer thicknesses and scattering properties in phantom media. The two-layered phantoms were made to be optically homogenous in which polystyrene beads (07307, Polysciences, Warrington, USA) were added into both the top and bottom layers of the phantoms at different concentration to achieve scattering coefficients of 0 cm^{-1} , 25 cm^{-1} , 50 cm^{-1} and 75 cm^{-1} at an excitation wavelength of 785 nm. These values were chosen in this study to cover the range of scattering coefficients of normal human skin at 785 nm¹²⁴. Raman spectra were measured using a micro-Raman spectrometer system (inVia, Renishaw, UK) coupled to a microscope (Alpha 300, WITec, Germany) in a backscattering geometry. Microscope objective with 20X magnification and an NA of 0.40 was used in this study. A 785-nm diode laser with a power of 50 mW on sample surface was used for excitation and the illumination time was 5 seconds per spectrum. A depth series measurement mode of the Raman system was selected in which the phantom was scanned vertically with a step size of 90 μm until the laser spot was 180 μm below the boundary of the two layers. Each scaling factor (α_{i-1} , $i = 1$,

2, ..., N-1) was calculated using the urea peak intensities measured from 90 μm above (CR_{i-1}) and beneath the boundary (CR_i) of a phantom for a given top layer thickness and scattering coefficient. The changes in the scaling factor upon different top layer thicknesses and scattering coefficients of phantom media were plotted in Fig. 4.12 (a) and (b) respectively.

Figure 4. 12 (a) Scaling factor versus top layer thickness for a range of scattering coefficients; (b) scaling factor versus scattering coefficient for a range of top layer thicknesses. The error bars indicate the standard deviations of the scaling factors computed from three independent measurements.

Fig. 4.12 (a) shows the changes in scaling factors when the thickness of the top layer increases from 180 μm to 720 μm in phantoms with four different scattering coefficients. In phantoms with top layer thickness of 180 μm , the scaling factors for different scattering coefficients are close to each other with values between 0.35 to 0.4. When the top layer thickness increases to 360 μm , the scaling factor of media with scattering coefficients of 50 cm^{-1} and 75 cm^{-1} increases to 0.45 which is approximately 0.05 higher than those in phantoms with scattering coefficients of 0 cm^{-1} and 25 cm^{-1} . In phantoms with top layer thickness of 540 μm , the scaling factor for 0 cm^{-1} remains unchanged while significant increment in scaling factor is observed in other phantoms. The scaling factor increases to 0.45 in medium with 25 cm^{-1} , 0.6 in phantoms with 50 cm^{-1} , and 0.62 in phantoms with 75 cm^{-1} . Further increment in the values of scaling factor is

observed in phantoms with top layer of 720 μm except for 0 cm^{-1} . Generally the scaling factor increases with the thickness of top layer in phantoms.

The changes in scaling factors when the scattering coefficients of phantoms increase from 0 cm^{-1} to 75 cm^{-1} are shown in Fig. 4.12 (b). When the scattering coefficient is 0 cm^{-1} , the scaling factor remains around 0.4 for all thicknesses of the top layer. When the scattering coefficient of the phantom increases to 25 cm^{-1} , the scaling factors of phantom with top layer thicknesses of 540 μm and 720 μm increase to 0.45 and 0.55 respectively while the scaling factors of the other two phantoms remain unchanged. In phantoms with scattering coefficient of 50 cm^{-1} and 75 cm^{-1} , similar trends are observed where the scaling factor increases to 0.45 for top layer thickness of 360 μm , 0.6 for a top layer thickness of 540 μm and 0.7 for a top layer thickness of 720 μm . Generally, the scaling factors increase when the scattering coefficient of the phantom increases. From Fig. 4.12 (a) and (b), we can see that the contribution of Raman signals from overlaying layers increases with the scattering coefficient and thickness of the overlaying layers. We have demonstrated that the progressive estimation method is capable of estimating the contribution of overlaying layers when performing subsurface optical measurements from optically scattering phantoms. Further works are needed to build up a comprehensive lookup table of scaling coefficients for more top layer thicknesses and scattering coefficients. The effect of numerical aperture of the focusing lens for excitation and signal collection and other factors such as the heterogeneity of scattering property in multi-layered phantom will be investigated to further refine the robustness of our method to be employed by other optical systems in a real application such as skin diagnosis.

In this study we have demonstrated the feasibility of a progressive estimation method to extract layer specific optical signals from an optically scattering phantom by

estimating the signals originated from all shallower layers and subtracting them away from the measured signals from the targeted depth. This method can be very useful to improving the depth sensitivity of any Raman microscope or optical system that employs the cone illumination and detection configuration as it involves no physical modification and requires only two measurements from two depths to extract layer specific optical signals. In the following chapters, an unsupervised approach will be investigated to reduce the signal contribution from the overlaying layers by employing a novel cone shell illumination and detection configuration. Note that by unsupervised approach, we mean that no information from other depths is needed to extract layer-specific information from the targeted depth of interest. The depth sensitivity of the novel cone shell configuration will be systematically evaluated in the next chapter.

Chapter 5 Monte Carlo Modeling of Depth Sensitive Optical Measurements and Its Phantom Validation

[Copyright Permission from: Y. H. Ong, C. Zhu and Q. Liu, "Phantom validation of Monte Carlo modeling for noncontact depth sensitive fluorescence measurements in an epithelial tissue model," *Journal of biomedical optics* **19**(8), (2014)]

5.1 Introduction

In the previous chapter, we have demonstrated that a cone shell configuration exhibits superior depth sensitivity than a conventional cone configuration in a phantom experiment. The cone shell configuration was achieved by inserting an aperture slider in the excitation and detection light pathways of a microscope objective lens to block the light which passes through the centre of the lens. A major drawback of this setup is that a large portion of the excitation light power was blocked by the aperture slider which resulted in a significant reduction in the detected fluorescence intensity. This issue has to be resolved as the optical signal originated from a biological tissue is inherently weak. Conversion of a full excitation beam into an annular excitation laser ring can get rid of the aperture slider while maintaining the full excitation power. The creation of a laser ring from a collimated laser beam involves additional optical components and the details will be discussed in the following section. Systematic investigation of various optical parameters is important to further improve the depth sensitivity of a cone shell configuration. However, experimental investigation and optimization of various optical parameters in the design of depth sensitive optical measurements in layered tissues would require a huge amount of time and resources. A computational method to model light transport in layered tissues using Monte Carlo simulations has been developed for decades to reduce the cost incurred during this process. In this chapter, we employed the Monte Carlo method to model the traditional cone and novel cone shell configuration and investigate the depth sensitivity achieved

by varying different optical parameters. Phantom experiments have been carried out to validate Monte Carlo modeling of fluorescence in a two-layered turbid, epithelial tissue model. The findings of this study and the development of the Monte Carlo method for non-contact setups provide useful insight and assistance in the planning and optimization of optical designs for depth sensitive fluorescence measurements.

Fluorescence spectroscopy has been widely explored for the detection of precancers and cancers in human epithelial tissues. Being an optical spectroscopic technique, fluorescence spectroscopy is capable of rapidly and non-invasively quantifying biochemical and morphological changes in human tissues due to the rich endogenous fluorophore contents in epithelial tissues that contain vital diagnostic information for the identification of tissue malignancy. Generally, an epithelial tissue consists of an outer epithelial layer that rests on the basement membrane and an underlying stromal layer. The distribution of these fluorophores in an epithelial tissue is depth dependent and can be affected by several factors, such as the age, menopausal status¹²⁸ and disease progression^{38, 129}. The optical scattering property of epithelial tissues further complicates the *in vivo* localization of these fluorophores. Thus, an optical setup of illumination and detection with excellent depth sensitivity is highly preferable to improve the diagnostic accuracy of this technique in epithelial precancer and cancer.

Depth sensitive fluorescence measurements have previously been demonstrated using contact fiber-optic based probe geometries by varying the source-detector separation³⁹, aperture diameter⁴⁰, and tilt angle of illumination and collection fibers⁴¹. However, a fiber-optic based probe requires contact with the sample surface and a past study has shown that the inconsistent pressure exerted on biological tissues can cause spectral distortion that severely affects the diagnostic accuracy of this technique⁴².

Besides that, the contact between the probe and the patient's skin especially on infectious sites may cause probe contamination and elevate the risk of disease transmission across patients. To overcome these complications, alternative non-contact measurement methods have been investigated by using lens-based setups. Bish et al.¹³⁰ performed non-contact diffuse reflectance measurements on tissue phantoms and human skin by using a lens based probe in an effort to eliminate diagnostic inconsistency due to the exertion of probe pressure. Mazurenka et al.¹³¹ demonstrated non-contact time-resolved diffuse reflectance measurements with a lens based setup, and laser scanning was used to achieve imaging. Generally, these lens-based setups use the combination of lenses to achieve the excitation and collection volumes that would form cones in an optically transparent medium. Even though the non-contact setup has the advantage to minimize the risk of disease transmission and inconsistent probe-sample pressure exertion, it suffers from limited sensitivity to subsurface fluorescence signals due to the signal contribution from shallower layers in a layered structure such as the epithelial tissue, which is similar to the non-contact fiber-optic based setup,.

Our group has previously demonstrated that a cone shell illumination configuration was able to improve the depth sensitivity of fluorescence signals from deep layers by reducing the contribution from shallower layer. We have experimentally investigated the cone shell illumination configuration implemented by multiple axicon lenses in a fluorescence study using a two-layered tissue phantom which mimicked the optical properties of human skin¹³². The details of this study can be found in Chapter 5. The results showed that the cone shell illumination configuration was able to detect fluorescence signals with a larger sensitivity to the deep layer and a larger range of sensitivity to the top and bottom layers than a conventional cone configuration

implemented by a convex lens. It has been demonstrated that the cone shell illumination configuration implemented by axicon lenses exhibits enhanced depth sensitivity compared to the cone configuration implemented by a convex lens. Further enhancement in depth sensitivity is expected through the systematic optimization of various optical parameters, such as the diameter, height and apex angle of axicon lens, lens-sample distance, and the outer and inner diameter of the cone shell illumination beam, etc. However, the experimental investigation of these parameters would require a huge amount of time and resources. For instance, an axicon lens with a fixed apex angle is needed to create a cone shell with a particular incident angle on the sample surface but it is not cost effective to get multiple expensive axicon lenses with a full range of different apex angles for optimization.

To effectively reproduce the experimental configuration of these optical measurements without incurring significant cost, the Monte Carlo method has been developed and employed in the past few decades, as a versatile computational tool to model light propagation in turbid tissue-like media. Optical configurations and light-tissue interactions (scattering, absorption and fluorescence) can be modelled to simulate light distribution in the numerical model of human tissues, which has been extremely useful in helping the design and optimization of various experiments and optical setups. Keijzer et al.¹³³ used the Monte Carlo method to simulate fluorescence in turbid media and investigated the effect of the geometry in the excitation light delivery and emission light detection on measured signals. Wang and Jacques's versatile Monte Carlo simulation package¹³⁴⁻¹³⁶ for the modelling of photon transport in multi-layered tissues has been publicized and received enormous attention. Welch et al.¹³⁷ have evaluated the effect of tissue optical properties and the geometry of tissue sample using Monte Carlo simulations. In another Monte Carlo simulation study, Qu

et al.¹³⁸ demonstrated that maximizing the overlap between the illumination and detection area can reduce the distortion in measured fluorescence signals due to variation in the tissue optical property with wavelength. In achieving depth sensitive fluorescence measurement, Zhu et al.¹³⁹ used Monte Carlo simulations to model different contact fiber-optic based probe geometries in fluorescence measurements from different depths in epithelial tissues. Recently, Zhu et al.¹⁴⁰ from the authors' group has simulated the cone and cone shell configuration implemented by a convex lens in a non-contact imaging geometry using the Monte Carlo method and the results showed that the cone shell configuration yields a larger sensitivity to fluorescence from deep layers.

Although the Monte Carlo method has been widely employed to study light propagation in turbid media, limited works on the experimental validation of Monte Carlo modeling have been reported. Pogue et. al. used the Monte Carlo method to study fluorescence measured from tissue samples using a fiber bundle with an individual fiber diameter of 100 μm , in which experiments were performed to verify the results. Liu et al.⁴¹ used Monte Carlo modelling to simulate fluorescence measured from turbid tissue phantoms by an angled fiber-optic probe and demonstrated that depth selectivity can be achieved by varying the illumination angle. The simulation results have been verified with experiments by using two-layered epithelial tissue phantoms. Liu et. al.¹⁴¹ have also used the Monte Carlo method to simulate fluorescence and diffuse reflectance values measured by several different fiber-optic probe geometries that were designed to sample small tissue volumes. They quantitatively compared the numerically simulated and experimentally measured results to validate the Monte Carlo model.

Even though there has been an increasing interest in using the Monte Carlo method to simulate fluorescence light transport in turbid media, most previous reports focused on using fiber-optic based probe geometries for illumination and detection. Investigation on non-contact lens-based probe geometries using the Monte Carlo method has been so far very limited. The goal of this study is to experimentally verify Monte Carlo modeling of fluorescence measurements involving non-contact lens-based probe geometries from turbid media with a layered structure. We have developed a Monte Carlo model to simulate different combinations of illumination and detection configurations, involving both the cone and cone shell geometry, on a tissue model. In particular, simulations and experiments were carried out to assess the depth sensitivity performance of different combination of illumination and detection configuration in a non-contact lens based probe geometry, implemented by axicon or convex lenses, in a two-layered turbid medium mimicking the optical properties of human epithelial tissue.

In this work presented, we numerically and experimentally investigated various combination of illumination and detection configurations. The simulation results are validated with the experimental results in terms of fluorescence intensities at the emission peaks and the depth sensitivity to a given layer. The experimental results provide useful insights to the change in depth sensitivity achieved using different types of lenses and illumination-detection geometry. The development and validation of this Monte Carlo code provides a fast, inexpensive, reliable and robust computational platform that can assist the planning and optimization of optical designs involving the cone or cone shell illumination and detection geometries prior to the physical development of an optical system for real experiments.

5.2 Experimental Setups

Figure 5.1 Schematic diagram of four illumination – detection configurations: (a) Cone – Cone, (b) Cone shell – Cone, (c) Cone shell – Cone, and (d) Cone shell – Cone shell. Configurations (a) and (b) are implemented using convex lens; configurations (c) and (d) are implemented using axicon lens. The solid arrow represents the excitation laser light while the dashed arrow represents the emitted fluorescence light.

In this study, we investigated four different illumination and detection configurations involving both the cone and cone shell geometries. The four illumination-detection configurations are shown in Fig. 5.1, where (a) represents a cone (illumination) - cone (detection) and (b) a cone shell - cone configuration implemented by a convex lens, (c) a cone shell - cone configuration and (d) a cone shell - cone shell configuration implemented by an axicon lens.

Figure 5.2 Schematic diagram of the probe configuration based on the combination of (a) convex lenses and (b) axicon lenses.

The non-contact probe for configurations (a) and (b) in Fig. 5.1 was coupled to a diode laser (iFlex-2000, Point Source Ltd., Hamble, UK) with a maximum output power of 50mW at 405nm, as shown in Fig. 5.2 (a). The output laser light with a beam diameter of around 1 mm was expanded using a 30x beam expander before passing through a 405 nm bandpass filter and then deflected by a dichroic mirror towards a convex lens ($f = 35$ mm) with a diameter of 25.4 mm, in which the lens was slightly overfilled. A piece of aluminium foil with a diameter of 23.4 mm was placed along the excitation light path, between the beam expander and bandpass filter, to create a cone shell illumination configuration as shown in Fig. 5.1 (b). The fluorescence signal was then collected through the same convex lens, which then passed through the dichroic

mirror and a long pass filter before being focused onto the core of a collection fiber, with a diameter of 400 μm and NA of 0.22, by a convex lens ($f = 35\text{ mm}$).

The non-contact probe for configurations (c) and (d) in Fig. 5.1 was constructed as shown in Fig. 5.2(b). For the configuration in Fig. 5.1(c), a collimated beam of around 3 mm in diameter was passed through a pair of axicon lenses with an apex angle of 140° to form an annular laser beam. The excitation beam was then passed through a bandpass filter and deflected by a dichroic mirror towards a third axicon lens with an apex angle of 110° , which formed a cone shell illumination configuration. The fluorescence signal was collected by the third axicon lens and focused onto the collection fiber as described above. The configuration in Fig. 5.1(d) was the same as the configuration in Fig. 5.1(c) except that a piece of circular aluminium sheet was placed on the third axicon lens to block the fluorescence signal that came back through the centre of the lens to create a cone shell detection configuration. The collected fluorescence signal was transmitted by the collection fibers to a Czerny-Turner type spectrograph (Shamrock 303, Andor Technology, Belfast, UK) and a research-grade CCD (DU920P-BR-DD, Andor Technology, Belfast, UK). The laser powers measured on the sample surface were around 2.5 mW for configuration (a) and (b) and 7 mW for configuration (c) and (d). The integration time for each measurement was one second in all experiments.

During measurements, the phantom was placed on a translational stage underneath the probe. For configuration (a) and (b), the probe-sample distance was varied to measure from different depths in the phantom by raising the stage towards the probe. The first measurement started at 0 mm where the focal spot of the excitation light was located on the surface of the phantom. Then, for every subsequent measurement, the stage was raised by 0.5 mm until it reached a depth of 4 mm beneath the surface. The

integration time for each spectrum was one second and the laser shutter was closed for ten seconds between each measurement to allow fluorescence recovery in case of the possible photo bleaching of fluorophores.

Figure 5.3 Schematic diagram of the axicon lens based setup. Axicon 1 and Axicon 2 are used to create collimated laser ring; Axicon 3 is used to create light with the cone shell geometry.

For configurations in Fig. 5.1(c) and 1(d), three plano-convex axicon lenses were used to map the axial dimension (depth) to the radial dimension (radius). An axicon lens can steer a collimated light at a fixed angle relative to the conical surface. Thus, by varying the radius of a collimated illumination light ring, it can focus the light at various depths therefore eliminating the need to move the probe up or down for depth sensitive measurements. In our setup, a plano-convex axicon lens (Axicon 3 in Fig. 5.3), with an apex angle (α_2) of 35° was used for illumination and detection. The relationship between α and β as labeled in Fig. 5.3 is given as:

$$\beta_N = \sin^{-1}(n \sin \alpha_N) - \alpha_N \quad \text{Eq. 1}$$

where n is the refractive index of the material of which axicon lens is made and N is the index of the angle.

All axicon lenses were made up of BK7 glass with a refractive index of 1.51 at 785 nm, and β_2 was calculated to be 25° . By using Pythagoras theorem, the maximum depth of focus from the apex of Axicon 3 (L_2) can be calculated as shown below:

$$L_2 = R \left(\frac{1}{\tan \beta_2} - \tan \alpha_2 \right) \quad \text{Eq. 2}$$

where R is the outer radius of the illumination light after axicon lens 2.

Equation 2 indicates that the depth of focus, L_2 , changes with the outer radius of the illumination light, R . In order for Axicon 3 to form a cone shell illumination configuration, a collimated laser ring is needed. The excitation laser ring can be created by passing a collimated laser through a pair of identical plano-convex axicon lenses ($\alpha_1 = 20^\circ$) with apexes facing each other, as shown in Fig. 5.2 and Fig. 5.3. The diameter of laser ring can be manipulated by changing the distance between the plano-convex axicon pair. The relation between the distance, L_1 , and the ring radius, R , can be deduced by using Eq. 3.

$$R = \frac{L_1}{\left(\frac{1}{\tan \beta_1} - \tan \alpha_1 \right)} \quad \text{Eq. 3}$$

So the depth of laser focus without changing the distance between the axicon lens' tip and the sample surface varies with the outer radius of the beam R , which can be in turn changed by varying the distance between Axicon 1 and Axicon 2 as shown in Table 5.1.

Table 5.1 Relationship between the separation distance between axicon lens 1 and axicon lens 2, L_1 (mm), the outer radius, R (mm), of the collimated excitation annular ring, the maximum depth of focus for the apex of axicon lens 3, L_2 (mm) and the actual depth of focus (mm) on tissue phantom.

L_1 (mm)	R (mm)	L_2 (mm)	Depth of focus (mm)
7.1	1.5	2.17	0
9.5	2.0	2.89	0.72
11.8	2.5	3.61	1.44
14.2	3.0	4.33	2.16
16.6	3.5	5.06	2.89
18.9	4.0	5.78	3.61
20.4	4.3	6.17	4.00

As the diameter of the excitation laser beam, D , incident on Axicon 1 was 3 mm, the smallest outer ring radius that can be created by Axicon 2 was 1.5 mm which corresponds to a minimum focal depth of 2.17 mm away from the apex of Axicon 3, as shown in the first row of Table 5.1. Therefore, in the experimental setup, the phantom was placed at a distance 2.17 mm below the probe tip (apex of Axicon 3), so that the focal spot of the laser is located exactly on the surface of the sample when the distance between Axicon 1 and Axicon 2 was 7.1 mm. Similarly other focal depth values were achieved according to Table 5.1. The probe-sample distance was fixed at 2.17 mm and remained unchanged throughout the experiments. Measurements were taken for a focal depth range of 4 mm with an increment of 0.5 mm in order to be consistent with the measurements of configuration (a) and (b).

5.3 Sample Preparation

A two-layered agar phantom was prepared according to the recipe and procedure published in an earlier report¹²³. The optical properties of the phantom was chosen to represent human cervical tissue¹⁴². The phantom was made in a cylindrical plastic petri

dish with a diameter of 30 mm, in which the top layer was made to be 500 μm while the bottom layer was made to be 10 mm to represent a semi-infinite medium. Two different fluorophores, flavin adenine dinucleotide (FAD) with peak emission at 530 nm and protoporphyrin IX (PpIX) with peak emission at 630 nm, were added into the top and bottom layers, respectively, for the ease of fluorescence discrimination from each layer. The concentration of FAD was 33.2 μM and the concentration of PpIX was 32.3 μM . Polystyrene spheres (07310, Polysciences, Warrington, Pennsylvania, USA) and Nigrosin (N4754, Sigma-Aldrich, St. Louis, Missouri, USA) served as the light scatterers and absorber. The concentration of polystyrene spheres needed to mimic the scattering coefficient of tissue at 530 nm was estimated using Mie theory and the concentration of nigrosin used to mimic the absorption coefficient of the tissue was calculated according to the measured extinction coefficient spectrum. The typical absorption and scattering coefficient spectra of epithelial tissue are different from those of the elastic scatterers (polystyrene spheres) and light absorbers (nigrosin) in shape from the excitation wavelength (405 nm) to the emission peak wavelengths (530 and 630 nm). Thus, the optical properties of the tissue phantom was chosen to match those of the epithelial tissue at the central wavelength, around 530 nm, in order to simulate and investigate the depth sensitive fluorescence measurements as close to the real epithelial tissue as possible. A piece of plastic wrap was placed between the top and bottom layer to prevent the diffusion of phantom contents across the two layers. The thickness of the plastic wrap was measured to be 10 μm and no fluorescence signal from the plastic wrap was observed in the experiment.

The optical properties of top and bottom layers of the tissue phantom at the excitation and emission wavelengths were listed in Table 5.2.

Table 5.2 Optical Properties (μ_s [cm^{-1}] and μ_a [cm^{-1}]) for Top and Bottom Layers for Tissue Phantom at the Excitation Wavelength and at the Peak Emission Wavelength of FAD and PpIX

Layer	Wavelength (nm)		
	405 (Excitation)	530 (FAD)	630 (PpIX)
Top	39.7, 1.2	34.2, 1.9	26.2, 1.8
Bottom	250.2, 1.4	215.1, 2.2	185.7, 2.1

5.4 Monte Carlo Modeling

The Monte Carlo method for simulating convex lens based light illumination and detection has been described in detail previously¹⁴³. The simulation of fluorescence is based on a method proposed by Liu et al earlier¹⁴¹. The Monte Carlo method for the simulation of axicon lens based light illumination and detection has never been reported before, and the details are described as follows.

The schematic of axicon lens based light illumination and detection is shown in Fig. 5.4. In the illumination module of the code, the radius of the axicon lens, the ring thickness, the apex angle, the thickness, and the distance between axicon lens and tissue surface can be specified according to the experimental configuration. These five parameters are denoted by R , d , α , d_0 , and d_I , respectively, in Fig. 5.4. The key step to be simulated for each launched photon is to find the incident location and the incident angle. The incident location of the photon on the surface of the tissue model can be determined in two steps. The first step is to find the incident location of the photon on the plano surface of axicon lens. For the collimated beam created by a fiber followed by a beam collimator, which is the case in this study, the spatial distribution of photons can be assumed to be Gaussian. So the radial position of a photon on the axicon lens surface can be sampled by

$$r = \sqrt{R^2 - d^2 * \varepsilon_r} \quad \text{Eq. 4}$$

where ε_r is a random number between 0 and 1 following Gaussian distribution. The azimuthal angle of the photon was sampled by

$$\theta = 2\pi\varepsilon_\theta \quad \text{Eq. 5}$$

where ε_θ is a random number uniformly distributed between 0 and 1. The Cartesian coordinates of the incident location on the top surface of the axicon lens in Fig. 4 (a) are then

$$x_{lens} = r * \cos \theta \quad \text{Eq. 6}$$

$$y_{lens} = r * \sin \theta \quad \text{Eq. 7}$$

Then in the second step, the incident location on the tissue surface can be easily obtained by applying Snell's law based on x_{lens} , y_{lens} , d_I and β that can be calculated from α . Once the photon is moved onto the tissue surface, Snell's law is applied to calculate the incident angles at the air-tissue interface due to the mismatch of refractive index between the air and tissue model. Based on the incident deflection angle and the incident locations on the tissue surface, it would be straightforward to find out the directional cosines.

Figure 5.4 Schematic of axicon lens based (a) light illumination; (b) light detection.

In the detection module as shown in Fig. 5.4(b), the parameters to be specified include the distance between the axicon lens and the convex lens used for focusing light onto the detection fiber, the radius and focal length of convex lens, and the size and numerical aperture (NA) of the detection fiber. The detection fiber, the convex lens and the axicon lens share the same common central axis passing through the origin of the Cartesian system. The tip of the detection fiber is located on the focal plane of the convex lens. The ray tracing procedure for a photon exiting from the tissue surface can be divided into seven steps described as follows.

- Step 1: find the intersection P between the exit path of the photon l_1 and the tapered surface of the axicon lens;
- Step 2: determine the directional vector of photon refraction on the tapered surface of the axicon lens labelled as l_2 by Snell's law;
- Step 3: find the intersection Q between l_2 and the top surface of the axicon lens.
- Step 4: determine the directional vector (l_3) of photon refraction on the top surface of the axicon lens by Snell's law;

Step 5: find the intersection R between l_3 and the convex lens;

Step 6: determine the directional vector (l_4) of photon refraction through the convex lens ;

Step 7: determine whether l_4 fulfill the conditions for the exit photon to be detected by the fiber.

The values of all simulated parameters were equal to those in our experimental study. A total of 0.5 million photons were used in each simulation and every simulation were repeated three times to calculate the standard deviation.

5.5 Data Analysis

Background subtraction was performed on all measured spectra. The background spectrum was acquired for every set of experiments with the excitation laser source switched off. The peak intensities at 530 nm and 630 nm were used for the plotting of FAD and PpIX fluorescence signal changes over a range of targeted depths in each configuration. The FAD peak intensity measured at every depth was divided by the maximum among these intensities to achieve normalization. Similarly, the PpIX peak intensity measured at every depth was divided by the maximum among these intensities to obtain the normalized PpIX intensity. This method of normalization facilitated the comparison of experimental results across different experimental setups by cancelling out the effect of different excitation laser powers on the sample due to the variation in optical setup. To compare the depth sensitivity achieved by each configuration, the sensitivities of measured fluorescence to the top and bottom layer as a function of the targeted depth were computed. The sensitivity to the top or bottom layer at a particular depth was computed by dividing the normalized FAD or PpIX intensity at this depth to the sum of the normalized FAD and PpIX intensities at the same depth.

In Monte Carlo simulations, the quantum yield of the fluorophore was always set to 1 and a combined absorption coefficient was used to account for two distinct absorption coefficients of the absorber and fluorophores. To compare the experimental and simulated results, the quantum yields of FAD and PpIX at both emission wavelengths had to be rescaled to the measured values and the absorption coefficients contributed separately by the absorber (Nigrosin) and fluorophores (FAD and PpIX) in the tissue phantom had to be accounted for in the Monte Carlo simulations. In Monte Carlo simulations, the absorption coefficient of the phantom model indicates the probability of an excitation photon being absorbed by Nigrosin or FAD/PpIX molecules, while the quantum yield of a fluorophore indicates the probability of an absorbed photon being converted to fluorescence light. The conversion of the absorption coefficient and rescaling of quantum yields from the experimentally measured values to the assigned value in simulations have been discussed in detail in the previous publication.¹⁴¹ The absorption coefficients of Nigrosin, FAD and PpIX at the excitation wavelength were experimentally measured using a UV-VIS spectrophotometer (UV-2450, Shimadzu Corp., Kyoto, Japan) at the individual concentrations used in the tissue phantom, respectively. For a photon being absorbed or scattered according to random sampling in the top layer, its probability of being absorbed by FAD molecules was calculated by taking the ratio of absorption coefficient of FAD to the sum of absorption coefficients of Nigrosin and FAD. Similarly, for a photon being absorbed or scattered according to random sampling in the bottom layer, the probability of excitation light being absorbed by PpIX was calculated by taking the ratio of absorption coefficient of PpIX to the sum of absorption coefficients of Nigrosin and PpIX.

To determine the quantum yield of FAD and PpIX in the tissue phantom for the purpose of simulation, the fluorescence spectra of FAD and PpIX were measured at very low concentrations first to minimise the secondary absorption effect of the fluorophore itself on emitted fluorescence. Background subtraction was performed on the spectra then their intensities were scaled up to the fluorophores concentrations used in the tissue phantom given that the fluorescence intensity is linearly proportional to the concentration of the fluorescence molecule in the concentration range in this study. The quantum yield of FAD at 530 nm was determined by taking the ratio of FAD fluorescence intensity to the sum of FAD and PpIX fluorescence intensities at 530 nm. The quantum yield of PpIX at 530 nm was computed by taking the ratio of PpIX fluorescence intensity to the sum of FAD and PpIX fluorescence intensities at 530 nm. The quantum yields of FAD and PpIX at 630 nm were determined similarly.

The simulated results, after the correction for both the absorption coefficient and quantum yield, were normalized in the same manner as in the processing of experimental data, as described earlier to facilitate the comparison of FAD and PpIX fluorescence trends between computational and experimental results. Then, the sensitivities to the top and bottom layer of each simulated configuration were computed using the same method as described above to further validate the Monte Carlo modelling that we have developed.

5.6 Results and Discussions

Figure 5.5 (a) Normalized FAD fluorescence and (b) normalized PpIX fluorescence measured from the tissue phantom using the Cone – Cone configuration as shown in Fig. 1(a), and the corresponding Monte Carlo simulation results. (c) Sensitivity to top layer and (d) sensitivity to bottom layer computed from the experimental results and simulated results. Focal depth refers to the targeted depth of focus beneath the phantom surface, which has been corrected for the refractive mismatch between the phantom and air.

Fig. 5.5 shows the trends of measured FAD and PpIX fluorescence and the sensitivities to the top and bottom layer in the cone – cone configuration for which the schematic is shown in Fig. 5.1(a). The experimental results are represented in solid lines and the simulation results are represented in dotted lines. The experiments were repeated three times at different locations on the same phantom, and the lens-sample distance was adjusted precisely using a translational stage. The standard deviations in the experimental results are small and not shown on the plot as they cannot be seen clearly at most points. In Fig. 5.5(a), it can be seen that the measured FAD

fluorescence rises slightly at the beginning and reaches its maximum at around 0.7 mm before decreasing gradually to around 0.22 at a focal depth of 5.5 mm. In Fig. 5.5(b), the measured PpIX fluorescence reaches the maximum at around 0.7 mm and then decreases slowly to 0.38 at a focal depth of 5.5 mm. The simulated fluorescence of FAD and PpIX are the highest also at around 0.7 mm and decreases to around 0.2 and 0.3, respectively, at focal depth of 5.5 mm. The overall trends in the simulated results are very similar to those of the measured results. The slight mismatch between the experimental and simulated results is observed at focal depths of 3 mm or longer could be potentially attributed to a few factors not well controlled. For example, water evaporation during phantom preparation or during measurement could cause changes in the optical properties and fluorophore concentrations of the phantom.

Fig. 5.5(c) and 5.5(d) show experimental and computational sensitivities to the top and bottom layers in the cone – cone configuration. The experimental sensitivity to the top layer is 0.49 at zero focal depth and reaches its peak of 0.5 at a focal depth of 0.7 before decreasing to 0.38 at a focal depth of 5.5 mm, while the experimental sensitivity to the bottom layer reaches the lowest value of 0.5 at a focal depth of 0.7 mm and increases to 0.62 at a focal depth of 5.5 mm. The simulated sensitivity to the top layer is the highest value of 0.51 at zero focal depth and decreases continuously to 0.39 at a focal depth of 5.5 mm while the simulation sensitivity to the bottom layer is the lowest value of 0.49 at zero focal depth and rises to the maximum of 0.61 at a focal depth of 5.5 mm. The overall trends of the sensitivities to the top and bottom layers in experimental results and simulated results agree with each other except at the first measurement point for zero focal depth. This difference is probably due to the uncertainty in the localization of zero focal depth in the experiment. As the position of zero focal depth in the experiment was determined by visually searching for the

smallest laser spot on the phantom surface, it might be slightly different from the actual position corresponding to zero focal depth that can be set precisely in Monte Carlo simulations.

Figure 5.6 (a) Normalized FAD fluorescence and (b) normalized PpIX measured from the tissue phantom using the Cone shell – Cone configuration as shown in Fig. 1(b), and the corresponding Monte Carlo simulation results. (c) Sensitivity to top layer and (d) sensitivity to bottom layer computed from the experimental results and simulated results. Focal depth refers to the targeted depth of focus beneath the phantom surface, which has been corrected for the refractive mismatch between the phantom and air.

Fig. 5.6 shows the trends of measured FAD and PpIX fluorescence and the sensitivities to the top and bottom layers in the cone shell – cone configuration implemented by a convex lens. In Fig. 5.6(a), it can be seen that the measured FAD fluorescence rises from 0.88 at zero focal depth and reaches its maximum at around 0.7 mm before decreasing gradually to around 0.11 at a focal depth of 5.5 mm. It

should be noted that FAD fluorescence in this configuration decreases faster after the maximum at a focal depth of 0.7 mm than those in the cone – cone configuration as shown in Fig. 5.5(a). This shows that the cone shell illumination geometry can be used to reduce the fluorescence contribution from shallower layers when performing deep measurements in a turbid medium, which agrees well with our previous report¹³². In Fig. 5.6(b), the measured PpIX fluorescence rises from 0.94 at zero focal depth to its maximum at a focal depth of 0.7 mm and then decreases slowly to 0.25 at a focal depth of 5.5 mm. The simulated FAD and PpIX fluorescence trends are in excellent agreement.

The experimental and simulated sensitivities to the top and bottom layers for the cone shell – cone configuration are shown in Fig. 5.6(c) and 5.6(d). Both experimental and simulated results show sensitivity to the top layer of 0.48 at zero focal depth and 0.5 at a focal depth of 0.7 mm. Then the experimental sensitivity to the top layer falls to 0.3 at a focal depth of 5.5 mm while the simulated sensitivity drops to 0.28 at the same focal depth. The sensitivity to the bottom layer in both experimental and simulated results is 0.52 at zero focal depth and drops to 0.5 at a focal depth of 0.7 mm before increasing to 0.70 and 0.72 at a focal depth of 5.5 mm, respectively. The trends in which the sensitivities to the top and bottom layers shift upon an increase in focal depth for the experimental and simulated results agree with each other and the relatively larger range of sensitivities achieved as shown in Fig. 5.6(c) and 5.6(d) compared to Fig. 5.5(c) and 5.5(d) suggest that the cone shell illumination configuration is able to reduce fluorescence from the overlaying layers in a turbid medium during deep measurements hence improving the contrast of the targeted subsurface fluorescence.

Figure 5.7 (a) Normalized FAD fluorescence and (b) normalized PpIX measured from the tissue phantom using the Cone shell – Cone configuration as shown in Fig. 1(c), and the corresponding simulated results. (c) Sensitivity to top layer and (d) sensitivity to bottom layer computed from the experimental results and simulated results. Focal depth refers to the targeted depth of focus beneath the phantom surface, which has been corrected for the refractive mismatch between the phantom and air.

Fig. 5.7 shows the trends of measured FAD and PpIX fluorescence and the sensitivities to the top and bottom layer in the cone shell – cone configuration as illustrated in Fig 5.1(c). The lens-sample distance was fixed throughout the measurements and the focal depth was varied by adjusting the distance between Axicon 1 and 2. Both of the two axicon lenses were mounted in a cage system, where the position of Axicon 1 was fixed and Axicon 2 can be slid towards or away from Axicon 1 then secured. The positions of Axicon 2 in the cage to achieve the nine focal depths were marked on the cage rods in order to reduce experimental variation in

repeated measurements. The standard deviations in the experimental results are slightly higher than those in the convex lens based setup as indicated by the error bars in Fig. 5.6. In Fig. 5.7(a), it can be seen that the measured FAD fluorescence rises from zero focal depth and reaches the maximum at around 0.7 mm. After that, the measured FAD fluorescence decreases to around 0.2 at a focal depth of 5.5 mm. In Fig. 5.7(b), the measured PpIX fluorescence rises from zero focal depth and reaches the maximum at around 1.3 mm. Then the measured PpIX decreases slowly to 0.37 at a focal depth of 5.5 mm. The trends in the simulated results are similar to the measured results.

Fig. 5.7(c) and 5.7(d) show the sensitivities of the cone shell – cone configuration to the top and bottom layers, respectively. The sensitivity to the top layer in both experiment and simulation are around 0.54 at zero focal depth. The sensitivity value drops as the focus moves towards the bottom layer and reaches around 0.31 in the experimental result and 0.29 in the simulated result at a focal depth of 5.5 mm. The sensitivities to the bottom layer in both sets of results are around 0.46 at zero focal depth and increases to around 0.69 in the experimental result and 0.71 in the simulated result at a focal depth of 5.5 mm.

Figure 5.8 (a) Normalized FAD fluorescence and (b) normalized PpIX fluorescence measured from the tissue phantom using the Cone shell – Cone shell configuration as shown in Fig. 1(d), and the corresponding Monte Carlo simulation results. (c) Sensitivity to the top layer and (d) sensitivity to the bottom layer computed from the experimental results and simulated results. Focal depth refers to the targeted depth of focus beneath the phantom surface, which has been corrected for the refractive mismatch between the phantom and air.

Fig. 5.8 shows the trends of measured FAD and PpIX fluorescence intensities and the sensitivities to the top and bottom layer for the cone shell – cone shell configuration as illustrated in Fig. 5.1(d). In Fig. 5.8(a), we can see that the measured FAD fluorescence rises a little initially and reaches the maximum at around 0.7 mm before decreasing to around 0.1 at a focal depth of 5.5mm with a sharp drop at focal depths between 0.7 and 2.7 mm. In Fig. 5.8(b), the measured PpIX fluorescence rises slightly from zero focal depth, which reaches the maximum at 0.7 mm and then decreases slowly to 0.24 at a focal depth of 5.5 mm. The trends in the Monte Carlo simulations are similar to the measured results. Fig. 5.8(c) and 5.8(d) show the close match between experimental and simulated sensitivities to the top and bottom layers in the cone shell – cone shell configuration. The sensitivity to the top layer in the

simulated result is around 0.53 at zero focal depth, slightly higher than that in the experimental result that is 0.51. Both sensitivities drop as the focal depth increases. The simulated sensitivity value reaches its minimum of 0.30 at a focal depth of around 4.8 mm and the experimental sensitivity reaches its minimum of 2.9 at a focal depth of 5.5 mm. The sensitivities to the bottom layer in experimental and simulated results are 0.49 and 0.47 at zero focal depth and increases to around 0.71 in the experiment and 0.7 in the simulations at a focal depth of 5.5 mm.

Figure 5.9 Comparison in the sensitivity to the (a) top layer and (b) bottom layer achieved by the four different optical configurations in the epithelial tissue phantom. AX refers to axicon lens; CL refers to convex lens; CS refers to cone shell configuration and C refers to cone configuration.

Fig. 5.9 shows the comparison in the sensitivity to the top and bottom layer achieved by the four different illumination-detection configurations as illustrated in Fig. 5.1. In Fig. 5.9(a), a decreasing trend in the sensitivity to the top layer in each configuration can be observed when the focal depth increases from 0 to 5.5 mm. Generally, when focusing at the surface of the phantom, axicon lens based setups show higher sensitivities to the top layer than convex lens based setups. This may be related to the fact that the axicon lens forms a focal line instead of a focal spot in case of the convex lens. When one focuses the centre part of the focal line formed by an axicon lens on the surface of the phantom, part of the focal line penetrates deeper into the

phantom and excites more signal from the illuminated regions. Thus, relatively more FAD fluorescence is excited at zero focal depth in an axicon setup, which elevated the sensitivity to the top layer. By comparing the sensitivities to the top layer achieved by the two cone shell – cone configurations implemented by different lenses, it can be seen that apart from the difference at small focal depths where the axicon lens based setup shows superior sensitivity, two setups have similar sensitivities to the top layer with a minimum sensitivity around 0.3 at a focal depth of 5.5 mm. In contrast, the cone – cone configuration achieved a minimum sensitivity to the top layer of 0.38 at a focal depth of 5.5 mm. The comparison demonstrates clearly that the cone shell illumination configuration can significantly reduce fluorescence contribution from the top layer when performing deep measurements. When a cone shell detection geometry is used in addition to a cone shell illumination configuration, as shown in the solid black line in Fig. 5.9(a), the minimum sensitivity to the top layer at a focal depth of 5.5 mm is further lowered by about 7% to 0.28. This result suggests that the cone shell – cone shell configuration is most suitable for depth sensitive measurements in a turbid medium. When the cone shell illumination configuration reduces the contribution of fluorescence from shallower layers, the cone shell detection configuration rejects out-of-focus fluorescence from those non-targeted depths that would otherwise reach the probe.

Comparatively, in Fig. 5.9(b), one can see an increasing trend in the sensitivity to the bottom layer in each configuration when the focal depth increases from 0 to 5.5 mm. Due to more fluorescence collected from the top layer by the axicon lens based setups at zero focal depth as explained above, the sensitivity to the bottom layer of the two axicon setups are lower than the convex lens based setups. At a focal depth of 5.5 mm, the cone shell – cone shell configuration has the highest sensitivity to the bottom

layer of 0.72, followed by the two cone shell – cone configuration with a sensitivity of around 0.7 and the cone – cone configuration with sensitivity of 0.62.

It is noted that the two cone shell – cone configurations implemented by the axicon lens and the convex lens, respectively, as represented by the grey lines and dashed lines in Fig. 5.9(a) and 5.9(b), demonstrate similar sensitivities to the top and bottom layers at all focal depths except at zero focal depth where the axicon lens based setup shows better sensitivities. However, the axicon lens based setup has its own disadvantages. The absolute sensitivity, i.e. the detected signal intensity divided by the incident light power density, achieved by an axicon lens setup is lower than that achieved by a convex lens setup. Given a similar focal depth and power density on the sample surface, the intensity of detected fluorescence signals using a cone shell – cone configuration implemented by the axicon lens is found to be around 1/5 of that detected using the same configuration implemented by convex lens. The decrease in the absolute sensitivity of an axicon lens setup can be attributed to the weaker focusing and signal collection power of the axicon lens due to its intrinsic structure. This is the price we have to pay in order to achieve higher depth sensitivity.

Apart from the structural difference in optical components, different optical configurations are found to have affected the absolute sensitivity of the optical system. In convex lens setups, the total fluorescence intensity detected using a cone shell – cone configuration is found to be around 5/6 of that detected using a cone – cone configuration with similar focal depth and power density. In axicon lens setups, the total fluorescence intensity detected using a cone shell – cone shell configuration is found to be around 2/3 of that detected by a cone shell – cone configuration with similar focal depth and power density. This shows that there is a trade off between depth sensitivity and absolute sensitivity of the system. While the rejection of out-of-

focus fluorescence signals by the cone shell configuration improves the depth sensitivity, the absolute sensitivity of the system degrades, which lowers the signal to noise ratio of the detected fluorescence. However, the decrease in absolute sensitivity can be compensated by increasing the laser power and/or acquisition time.

For fluorescence measurements from the superficial region of a tissue model, the axicon lens based setups would require considerably higher laser power and acquisition time than the convex lens based setups to achieve a similar signal to noise ratio. Moreover, the formation of an elongated focal line by the axicon lens can affect the axial resolution of the measurements compared to those achieved by a convex lens where a more confined laser focus can be formed. Nevertheless, the axial resolution of axicon lens based setups can be improved by minimising the length of the focal line formed, by using a thinner excitation ring (smaller d as shown in Fig. 5.3). Theoretically, the excitation ring thickness, d , is half of the incident laser beam diameter, D , as shown in Fig. 5.3. A pair of axicon lens with identical apex angle, Axicon 1 and Axicon 2, can steer the angle of the excitation beam, splitting it into a collimated annular ring with a thickness equal to half of the original beam diameter. Unfortunately, a portion of the excitation beam can pass through (instead of being refracted) the centre of the axicon lens in its original direction and illuminate the sample in our experiment due to the imperfection at the axicon lens's tip. Therefore, in the experiments of this study, a relatively larger excitation laser beam diameter of 3 mm was used to minimize the influence of light passing through the centre of the axicon lens on the measured results. Consequently, a longer laser focal line was formed that reduces the axial resolution and focusing power of the axicon lens based setups. The imperfect tips of the axicon lenses could be one of reasons for the slight

deviation between the experimental and simulated results as shown in Fig. 5.7 and Fig. 5.8.

Although the convex lenses are superior to the axicons in term of the power of light focusing and signal collection, it requires the lens-sample distance to be altered in order to measure from different depths in a sample. In many clinical applications such as skin cancer screening, a device without the need of precisely tuning the distance to the target is preferable as it offers physician flexibility to measure from various parts on the body and save the time of fine tuning. The difficulties in varying the device-sample distance accurately and maintaining that distance throughout measurements have limited the use of convex lens based setups for depth sensitive measurements in such clinical application. Axicon lens based setups for depth sensitive measurements without the need to move any component(s) up and down will bring significant convenience in these clinical applications, which could reduce the measurement uncertainty induced by the inconsistency in the device-sample distance compared to convex lens based setups.

5.7 Conclusion

In this study, we have investigated the depth sensitivity of four illumination and detection configurations involving the conventional cone and novel cone shell geometry implemented by a convex lens or an axicon lens, in a turbid medium. The results show that a cone shell illumination configuration was able to reduce the fluorescence contribution from the overlaying layers when performing deep measurements as compared to a cone illumination configuration. A cone shell detection configuration can reject undesired fluorescence from off-focus regions, hence, giving the best depth sensitivity in conjunction with a cone shell illumination configuration. The use of convex lens or axicon lens in implementing the illumination-

detection configurations has no profound effect on the depth sensitivity. However, both of the lenses are suitable for different clinical applications due to their own pros and cons and none of them is superior over the other. To facilitate the optimization of optical system, we have developed a Monte Carlo simulation to model the convex lens and axicon lens in implementing the cone and cone shell illumination and detection configurations. The simulation results were validated against the experimental results in term of fluorescence intensity and depth sensitivity. This Monte Carlo code will be a useful tool that helps in planning and optimization of optical designs involving cone or cone shell configurations and axicon or convex lenses prior to the real experimental study.

Chapter 6 Axicon Lenses Based Cone Shell Configuration for Depth Sensitive Measurements in Turbid Media

[Copyright permission from: Y. H. Ong and Q. Liu, "Axicon lens-based cone shell configuration for depth-sensitive fluorescence measurements in turbid media," *Optics Letters* **38**(15), 2647-2649 (2013)]

6.1 Introduction

In this chapter, we will demonstrate a depth sensitive handheld probe design in a fluorescence study. Ultraviolet-visible fluorescence spectroscopy has been widely explored in the detection of precancers in human epithelial tissues. Vital diagnostic information about the tissue morphological and biochemical changes can be easily extracted from various fluorophores present in epithelial tissues. However the distribution of endogenous fluorophores is affected by the progression of disease state and various other factors⁴¹. Hence, a depth sensitive probe that can measure fluorescence spectra as a function of depth will enhance the diagnostic performance of this technique. Currently, depth sensitive fluorescence measurements can be achieved in two approaches, i.e. the fiber-optic based contact setup and the lens based non-contact setup. Fiber-optic setups achieve depth sensitive measurements by varying the source-detector separation¹⁴⁴, aperture diameter⁴⁰ and (or) angle of illumination and collection fibers⁴¹. Lens based setups¹⁴⁵ use a single lens or a combination of lenses to achieve a cone configuration, in which both the excitation and emission volumes in an optically transparent medium would form light cones. One weakness of this setup is the limited sensitivity to deep subsurface layers due to the dominance of fluorescence from shallower layers. A cone shell configuration has been previously proposed¹⁴⁵ in a numerical study to yield larger depth sensitivity to deep layers than the cone configuration in diffuse reflectance measurements from a squamous cell carcinoma numerical model. The cone shell configuration was achieved by placing a ring mask on top of an imaging lens. Therefore it would be required to vary the distance between the imaging lens and the tissue sample in order

to achieve depth sensitive measurements, which induces uncertainty in optical coupling and great inconvenience in clinical measurements.

In previous chapter, we have introduced a novel cone shell illumination and collection configuration to overcome the weaknesses of the cone configuration in limited depth sensitivity to deep layers. Particularly, we demonstrated the use of a combination of three axicon lenses to eliminate the need of altering the lens-sample distance in achieving depth sensitive measurements. In this study, we integrated the cone shell illumination configuration implemented by combination of axicon lenses into a handheld probe design. A handheld probe design is useful for various clinical applications especially for skin diagnosis as it offers flexibility to the doctors to measure on various sites on the body conveniently. Besides that, a handheld design with reduced probe size is superior to a conventional microscopy setup in term of portability and mobility as this system can be easily moved around especially towards handicapped or bed-ridden patients in the wards. This will help saving time and human resources in transferring patients around in the hospital and effectively improving the quality of care management.

There are a few challenges in developing a handheld probing device. First, the size and weight of the entire system has to be reduced so that it can be moved or transported conveniently as a portable bedside tool. Second, the probe has to be small and compact so that it can be flexibly used to measure from various skin sites on the patient's body easily while providing a robust framework to maintain the precision of all components. Third, the light path of the probe must be shielded to ensure the safety of the users and patients and to minimize the interference from the ambient light. Fourth, the probe must contain minimal mechanical moving part in order to improve the measurement consistency. We have taken into consideration of all these challenges and have built a prototype of handheld probe with the novel cone shell illumination configuration.

A fluorescence phantom study was conducted to evaluate the performance of the probe in comparison to a conventional fluorescence microscope. Although a fluorescence study was performed, it should be noted that the same configuration could be also applied in other optical measurements such as diffuse reflectance or Raman measurements.

In the following sections, the details for the optical setup of our proposed system will be described. Then, the advantage of this handheld probe with cone shell configuration in term of depth sensitivity will be compared to a conventional microscope with cone configuration experimentally.

6.2 Experimental Setups

Figure 6.1 Schematic diagram of (a) the cone illumination and collection configuration based on a microscope objective lens, and (b) the cone shell configuration based on the combination of axicon lenses.

We constructed a fluorescence probe with the cone illumination and collection configuration as shown in Fig. 6.1(a). The probe was coupled to a diode laser (iFlex-2000, Point Source Ltd., Hamble, UK) through a single-mode fiber with a maximum output power of 30 mW at 405 nm. The output laser light was collimated using a convex lens ($f = 50.0$ mm) to achieve a beam diameter of 6 mm before passing through a 405 nm bandpass filter, and deflected by a dichroic mirror towards a microscope objective lens with 10X magnification power. The laser beam slightly overfilled the back aperture of the microscope objective lens which was approximately 5 mm in diameter to achieve a cone illumination configuration with a numerical aperture of 0.25. Fluorescence signals excited by the focused laser light were collected through the same objective lens, which then passed through a long pass filter before being focused onto the tip of a collection fiber with a core diameter of 400 μm and an NA of 0.22.

In the cone shell configuration, a collimated light beam of 3 mm in diameter from the same laser source as in the cone configuration was passed through a pair of axicon lenses (Altechna Co. Ltd., Vilnius, Lithuania), i.e. axicon 1 and axicon 2, with an identical apex angle (140°) to create a collimated annular laser ring as shown in Fig. 6.1(b). The laser ring was then focused onto the sample by the third axicon lens, i.e. axicon 3, with an apex angle of 110° , forming the cone shell geometry. The fluorescence signal will come back through axicon 3 and a long pass filter, and then be focused onto the same collection fiber as in the cone configuration. The fluorescence signals transmitted by the collection fiber in both configurations were coupled to a Czerny-Turner type spectrograph (Shamrock 303, Andor Technology, Belfast, UK) equipped with a holographic grating (1200 grove/mm) and a research-grade CCD (DU920P-BR-DD, Andor Technology, Belfast, UK), which yields a spectral resolution of 0.1 nm. The integration time for this experiment was always one second.

The laser powers on the sample were measured to be 10 mW and 7 mW for the cone and cone shell configurations, respectively.

6.3 Sample Preparations

A two-layered agar phantom was fabricated to mimic the stratified structure of human skin according to the recipe and procedure published in an earlier report.¹²³ The thickness of the top layer was 1 mm and the thickness of the bottom layer was 10 mm whereas the lateral dimension of both layer was made greater than 30 mm in diameter to represent a semi-infinite medium. Two different endogenous fluorophores, flavin adenine dinucleotide (FAD) and protoporphyrin IX (PpIX), were added to the top and bottom layer of the phantom, to discriminate fluorescence from two layers, which is facilitated by the non-overlapping emission peaks of FAD and PpIX at 525 nm and 630 nm, respectively. A concentration of 33.2 μM for FAD and a concentration of 32.3 μM for PpIX were used so that the intensities of both emission peaks fell within the same order of magnitude for the ease of data analysis. A piece of plastic wrap was placed between the two layers to prevent the diffusion of fluorophores between layers. Intralipid 20% (Fresenius Kabi, Bad Homburg, Germany) was added into each layer at a different concentration to mimic the light scattering properties of the epidermis and dermis so that the reduced scattering coefficient, μ'_s , matched the published value at 525 nm.¹⁴⁶ The optical properties of the phantom (μ'_s and μ_a) at the excitation wavelength (405 nm) and peak emission wavelengths of PpIX (630 nm) were estimated by scaling to the corresponding value reported elsewhere¹⁴⁷ and were listed in Table 6.1.

A range of apparent focal depths were achieved by varying the lens-sample distance in the cone configuration as shown in Fig. 6.1(a). A similar focal depth range was achieved by varying the distance between axicon lens 1 and 2 in the cone shell configuration as shown in Fig. 6.1(b), which minimized the variation in optical coupling. The actual focal depths inside

tissue phantoms were estimated by correcting for the refractive index mismatch between the phantom and air as described by Everall¹²⁵ based on a refractive index of 1.364 for the phantom and a refractive index of 1 for air at 405 nm.¹⁴⁸

Table 6.1 Optical Properties (μ'_s [cm^{-1}] and μ_a [cm^{-1}]) of the Top and Bottom Layers for Tissue Phantom at the Excitation Wavelength and at the Peak Emission Wavelengths of FAD and PpIX. μ'_s refers to the reduced scattering coefficient; μ_a refers to the absorption coefficient.

Layer	Wavelength (nm)		
	405 (Excitation)	540 (FAD)	630 (PpIX)
Top	80, 0.09	63, 0.03	55, 0.10
Bottom	51, 0.06	40, 0.02	35, 0.06

6.4 Results and Discussions

Figure 6.2 Fluorescence spectra measured using the cone shell configuration from the two-layered phantom at a range of focal depths corrected for refractive index mismatch. The legend indicates the corresponding actual focal depth of each spectrum. The abbreviation “c.u.” refers to the calibrated unit.

Fig. 6.2 shows the fluorescence spectra measured at a range of actual focal depths from the two-layered phantom using the cone shell configuration as shown in Fig. 1(b). FAD fluorescence peak intensity (at 525 nm) gradually increases from the surface (0 mm) until reaching the maximum at a depth of 1.3 mm and decreases noticeably from 1.3 mm to 5.2 mm. PpIX fluorescence peak intensity (at 630 nm) also increases as the focal depth increases from 0 mm to 2.6 mm and decreases as the focal depth increases further. This demonstrates that

cone shell configuration using axicon lenses is capable of performing depth sensitive fluorescence measurements without altering the lens-sample distance. To compare the sensitivity improvement achieved by this approach, we have computed the sensitivity of measured fluorescence to the top and bottom layer at all depths for both the cone and cone shell configurations as follows. First, the intensities of FAD and PpIX peaks were normalized to their maxima among the measurements at all depths, respectively. Then, the sensitivity to the top layer was obtained by dividing the normalized FAD peak intensity at one depth to the sum of the normalized peak intensities of both fluorophores at that particular depth. Similarly, the sensitivity to the bottom layer was obtained by dividing the normalized PpIX intensity at one depth to the sum of the normalized intensities of both fluorophores at the same depth.

Figure 6.3 Sensitivity to the (a) top layer and (b) bottom layer as a function of the actual focal depth, which has been corrected for refractive index mismatch between the sample and air.

Fig. 6.3 compares the sensitivity of measured fluorescence to the top and bottom layers between the cone and cone shell configurations. In Fig. 6.3 (a), it can be seen that the sensitivity to the top layer for the cone configuration, increases from 0.51 at the surface and reaches the maximum of 0.53 at a depth of 0.98 mm before decreasing to 0.40 as the focal depth increases to 6.5 mm. The sensitivity to the top layer for the cone shell configuration is ranged between 0.52 to 0.53 from the surface to a depth of 1.3 mm and it decreases to 0.32 at a depth of 6.5 mm. The maximum sensitivities of both the cone and cone shell configuration to the top layer are around 0.53 but the maximum location of the cone configuration has a narrower peak at 0.98 mm close to the actual thickness of the top layer. This can be explained by the difference in the focal spot size between two configurations. The microscope objective lens in the cone configuration formed a tiny focal spot of 20.9 μm in diameter which was much smaller than the top layer thickness. Different from that, the axicon lens formed an elongated focal line of around 1.7 mm in the axial dimension, which is estimated using Pythagoras theorem given that the thickness of the laser ring incident on aixcon 3 was measured to be 0.8 mm. Therefore the depth resolution of the cone configuration is superior to that of the cone shell configuration. In contrast, it can be seen that the minimum sensitivity to the top layer for the cone shell configuration, which is 0.32 at a focal depth of 6.5 mm, is lower than that for the cone configuration, which is 0.4 at the same focal depth. Therefore the range of the sensitivity to the top layer achieved with the cone shell configuration is larger than that with the cone configuration.

In Fig. 6.3(b), it can be seen that the maximum sensitivity to the bottom layer for the cone configuration, which is 0.60 at a focal depth of 6.5 mm, is considerably lower than that for the cone shell configuration, which is 0.68 at the same focal depth. It is noted that this depth corresponds to the minimum sensitivity to the top layer in Fig. 6.3(a). The bottom layer

fluorescence contrast of cone configuration was low due to the dominance of top layer fluorescence even though the laser intensity of the illumination cone base is much lower at the top layer than that of the illumination cone tip at greater depth. This observation can be attributed to the high possibility that the cone shell configuration effectively reduced the illumination volume near the surface when focusing on a deep layer due to the absence of the light core in the volume. As a consequence, the contribution of fluorescence from the top layer was effectively weakened, which resulted in a higher maximum sensitivity to the bottom layer. Moreover, the range of the sensitivity to the bottom layer achieved with the cone shell configuration is also larger than that with the cone configuration.

It should be noted that conventional microscope objective lens has better focusing power and light collecting efficiency than axicon lens as the magnitude of fluorescence detected by the CCD using microscope objective lens setup was stronger than the latter using similar excitation laser power. However, due to the ability of axicon lens setup in enhancing the contrast ratio of deep fluorescence and in eliminating the need of altering lens-sample distance when performing deep measurement, our axicon lens based cone shell light illumination and collection configuration would improve the performance and consistency of subsurface spectral acquisition in various clinical setting.

6.5 Conclusion

In this study, we have experimentally demonstrated that a handheld probe with cone shell configuration which involves multiple axicon lenses detects fluorescence with a higher sensitivity to the bottom layer and a larger range of sensitivity to either the top or the bottom layer in a two-layered tissue phantom mimicking human skin. Furthermore, these can be achieved without altering the distance between the imaging lens and the sample in the cone

shell configuration, which minimizes inconsistency in optical coupling and bring great convenience in potential clinical measurements.

Even though the relative movement between the probe and sample surface is successfully eliminated in this proposed system, mechanical movements of the axicon lens pair is still required to vary the focal distance of this probe. Accurate control of the displacement of the axicon lens is important to ensure the consistency of the focal depth achieved by the probe. Besides that, any mechanical movement of optical component in the probe may introduce uncertainties and inconsistency to the measurements. Thus, in the next chapter, we will explored the use of a collection fiber assembly to eliminate the need of changing the distance between the axicon lens pair in varying the focal depth of the probe. In addition of removing unwanted mechanical movement of optical components, the collection fiber assembly offers the capability to measure optical signals from multiple focal depths in one single acquisition. This will improve the speed of acquisition and offer more conveniences to various clinical settings. The details of the enhanced version of the handheld probe will be described in the next chapter.

Chapter 7 Fast Depth-sensitive Measurements in Turbid Media Using Collection Fiber Assembly with Cone Shell Configuration

[Copyright permission from: Y. H. Ong and Q. Liu, "Fast depth-sensitive fluorescence measurements in turbid media using cone shell configuration," *Journal of biomedical optics* **18**(11), (2013)]

7.1 Introduction

In Chapter 6, we have demonstrated a handheld probe design with cone shell configuration to achieved depth sensitive fluorescence measurements in turbid media. The probe shows higher sensitivity to the bottom layer and a larger range of sensitivity to either the top and bottom layer in a two-layered agar phantom mimicking the optical properties of the human skin. Particularly, a combination of three axicon lenses was used to eliminate the need of moving the probe up and down to measure from different depths. Even though there is no relative movement between the probe tip and sample surface, adjustment of the relative position between the axicon lens pair is required in order to vary the focal distance of the probe and this may also cause uncertainties and inconsistencies to the optical measurements. Besides that, the proposed system allows only one spectrum to be measured for each acquisition. A fast and consistent optical probe with no mechanical moving part is highly desirable in a clinical setting to reliably obtain useful diagnostic information from the tissues. In this chapter, we improve the handheld probe as described in chapter 6 in term of measurement consistency by removing unwanted mechanical moving parts in the optical system, and in term of acquisition speed by enabling multiple spectra to be measured from different depths in single acquisition. The enhancement in measurement consistency and acquisition speed of the new probe design will improve the diagnostic performance and provide greater conveniences to doctors in clinical measurements. The performance of the improved version of handheld probe will be evaluated in a fluorescence study similar to Chapter 6.

Autofluorescence spectroscopy in the ultraviolet/visible spectrum offers a non-invasive and effective approach for the detection of various epithelial cancers and precancers due to the rich endogenous biological fluorophores content in epithelial tissues that carry vital diagnostic information. As the depth distribution of these fluorophores is dependent on various factors such as age, menopausal status¹²⁸, and progression of disease state^{38, 129}, the excellent capability of performing depth sensitive fluorescence measurements in epithelial tissues will effectively improve the diagnostic performance of this technique. Achieving high depth sensitivity to a specific subsurface region in human epithelial tissue is a great challenge as photons will be quickly scattered when entering a tissue that is a diffusively scattering medium. Furthermore, the dominance of fluorescence signals from overlying layers greatly reduces the contrast of fluorescence signals originated from the subsurface region of interest, thus limiting the diagnostic performance of this technique. To overcome this limitation, we have previously introduced a non-contact axicon lenses based setup with a cone shell configuration that exhibited enhanced depth sensitivity to subsurface layers and a larger range of sensitivity to both the top and bottom layers in an epithelial tissue phantom than that of a conventional convex lens based setup with a cone configuration¹⁴⁹. Moreover, the axicon lenses based setup eliminates the need of moving the objective lens up or down, which is normally needed in a cone setup, to achieve depth sensitive measurements, which effectively improves the consistency of optical coupling thus would be preferred in a clinical setting. However, depth sensitive measurements using this setup can be time inefficient as it still requires the change in the distance between two axicon lenses in the setup.

In this study, we improve the spectra acquisition speed of the axicon lenses based setup by incorporating five rings of collection fibers into the detection configuration, which enables the collection of optical spectra from five different depths simultaneously. The new setup gets rid of the mechanical moving part consisting of two axicon lenses that are required

to achieve depth sensitive measurements. The performance of this improved setup is evaluated in the same manner as in Chapter 6.

7.2 Experimental Setups

Figure 7.1 Schematic diagram of experimental setup : EF, excitation fiber; CL, collimating lens; LLF, laser line filter; DM, dichroic mirror; AL, axicon lens; LPF, longpass filter; IL, imaging lens; TP, tissue phantom; CFA, collection fiber assembly.

The fluorescence fiber assembly with five rings of collection fibers was constructed as shown in Fig. 7.1. A 405 nm diode laser (iFlex-2000, Point Source Ltd., Hamble, UK) coupled to a single-mode fiber was used to deliver excitation light with a maximum output power of 30 mW. The output laser light was collimated using a convex lens ($f = 75.0$ mm) to achieve a beam diameter of 9 mm. Then, the beam passed through a 405 nm bandpass filter and a dichroic mirror and before focusing on the sample by an axicon lens, with an apex angle of 110° . The axicon lens was fixed at a distance of 0.89 mm above the sample surface. The fluorescence signal was collected through the same axicon lens and passed through a long pass filter before being imaged onto the tip of a custom designed fiber assembly (Leoni (SEA) Pte.

Ltd., Singapore) by a convex lens ($f = 35.0$ mm). The distance between this imaging lens, IL, and the tip of the collection fiber assembly end B was adjusted to be 28.5 mm.

Figure 7.2 Factory drawing of the collection fiber assembly.

The fiber assembly was comprised 91 optical fibers in which 48 was live fibers and 43 was dead spacer fibers with core/cladding/coating diameters of 100/110/125 µm and a numerical aperture of 0.22. The length of the fiber assembly was 1.5 meter and both ends of the cable were connected to a ferrule as shown in Fig. 7.2. The dimension of the ferrule at end A was 12 mm in diameter and 50 mm in length while the dimension of the ferrule at end B was 13 mm in diameter and 50 mm in length. The material of the ferrules was stainless steel. The tubing material was PVC that is compatible with ethylene oxide (gas sterilization) and Cydex and the diameter was around 7.62 mm.

Figure 7.3 Cross sectional view of the collection fiber assembly on end A (CFA-A) and end B (CFA-B). Color dots represent live fibers while black dots represent dead fibers for spacing.

The fibers were centered in the ferrules and the live fibers were represented by colored dots while the dead fibers were represented by black dots in Fig. 7.3. Live fibers at end A of the fiber assembly were symmetrically arranged into five rings surrounding a central spacer fiber, in which the number of collection fibers was 6, 6, 12, 12 and 12, respectively, from the inner to the outer ring.

At end B of the fiber assembly, these live fibers were arranged into five blocks each with six rows of fibers, and any two adjacent blocks of fibers were vertically separated by 500 μm . Dead fibers were used to fill the gap between the blocks and were not shown in Fig 7.3 and Fig 7.4. The first two blocks had one fiber in each row while blocks 3, 4 and 5 had two fibers in each row, as shown in Fig. 7.4. The width of the slit is two times the diameter of an individual collection fiber or $125 \mu\text{m} \times 2 = 250 \mu\text{m}$ and the height of the slit is $5 \times 0.75 \text{ mm} + 4 \times 0.50 \text{ mm} + 2 \times 0.33 \text{ mm} = 6.4 \text{ mm}$. The height of the slit is designed to

be 6.4 mm in order to take full advantage of the entire height of the spectrometer CCD chip which is also 6.4 mm. From the top to the bottom of Fig. 7.4, the five blocks corresponded to rings 1, 2, 3, 4 and 5 in the order from the inner ring to the outer ring.

Figure 7.4 Arrangement of optical fibers on the collection fiber assembly end B (CFA-B).

Figure 7.5 (a) Schematic diagram showing the pixel number and dimension of each pixel on the CCD chip of Andor Spectrometer; (b) Schematic diagram showing the dimension of the entire CCD chip of Andor spectrometer. The five light grey areas represent the regions where detected photons are binned vertically to produce five spectra while the dark grey areas represent the position of the five fiber blocks on CFA-B mapped on the CCD chip.

The fluorescence signals collected by the fiber assembly were directed to a Czerny-Turner type spectrograph (Shamrock 303, Andor Technology, Belfast, UK), which was equipped with a holographic grating (1200 grove/mm) and a research-grade CCD (DU920P-BR-DD, Andor Technology, Belfast, UK) that yields a spectral resolution of 0.1 nm. The input slit of the spectrometer was set to be 250-μm wide so as to allow the signals from fiber assembly end B to enter. The CCD chip consisted of 1024 X 256 pixels and the dimension of each pixel was 25 μm X 25 μm as shown in Fig 7.5(a). The dimension of the entire CCD was 25.6 mm X 6.4 mm and it was horizontally divided into 5 regions, represented by light grey areas as shown in Fig 7.5(b). These five regions were spaced apart vertically so that each region covered one block on the fiber assembly end B, represented by dark grey regions as shown in Fig. 7.5(b). Photons reaching each CCD region were binned vertically to produce five spectra corresponding to five individual fiber blocks for each measurement. The

integration time for this experiment was always 0.5 second. The laser powers incident on the sample were measured to be 15 mW.

A two-layered agar phantom was fabricated using the recipe and procedure reported elsewhere¹²³. FAD was added to the top layer at a concentration of 33.2 μM while PpIX was added to the bottom layer at a concentration of 32.3 μM . FAD and PpIX were chosen as the fluorophores for this study because they are intrinsic biological fluorophores that can be found inside tissues¹⁵⁰, with non-overlapping emission peaks at 525 nm and 630 nm, respectively. The dimension and optical properties of the phantom were made identical to our previous work as described in Chapter 6.

During measurements, the collected signals that reached the imaging lens were focused onto the tip of fiber assembly end A with an approximate demagnification factor of 5.4. The axicon lens was lifted to 0.89 mm above the sample so as to prevent the superficial fluorescence signals from reaching the central dead fiber on the fiber assembly end A. Each ring of collection fibers on the collection fiber assembly corresponded to a different targeted depth in the tissue phantom. The beam deviation angle, β given by:

$$\beta = \sin^{-1}(n \cdot \sin \alpha) - \alpha \quad (1)$$

where the refractive index, n , of the axicon (N-BK7 glass) is 1.53 at 405 nm¹⁵¹.

The corresponding depth in the phantom for each fiber ring was computed based on the equations shown in Fig. 7.6 which were derived from Pythagoras Theorem. The corresponding depths of measurements for each ring of fibers, L_2 , were then corrected for refractive index mismatch between the tissue phantom and the air as described by Everall¹²⁵, and were shown in Table 7.1. The calculation was based on the refractive indices of air and intralipid 20% at 405 nm, which are 1 and 1.364¹⁴⁸, respectively.

$$\tan \alpha = \frac{L_1}{D}$$

$$\tan \beta = \frac{D}{L_1 + L_2}$$

$$L_1 = D * \tan \alpha \quad \text{---- (1)}$$

$$D = \tan \beta * (L_1 + L_2) \quad \text{---- (2)}$$

Solving (1) and (2), we get :

$$L_2 = D \left(\frac{1 - \tan \alpha \tan \beta}{\tan \beta} \right)$$

Figure 7.6 Schematic diagram of an axicon lens and equations employed to compute the corresponding focal depth, L_2 from light beam with a diameter of D .

Table 7.1 Radial distance of each ring of collection fibers from the centre of probe, radius of light beam on the plano side of axicon lens, the corresponding depth in phantoms before and after offset, and corresponding depth offset and correction for refractive index mismatch.

Ring	Radius on probe (mm)	Radius on axicon, D (mm)	corresponding depth before offset, L_2 (mm)	corresponding depth after offset (mm)	Corresponding depth after offset and refractive mismatch correction (mm)
1	0.125	0.675	0.89	0	0
2	0.25	1.35	1.78	0.89	1.16
3	0.375	2.025	2.67	1.78	2.32
4	0.5	2.7	3.56	2.67	3.48
5	0.625	3.375	4.46	3.57	4.63

7.3 Results and Discussions

Figure 7.7 Fluorescence spectra measured using different rings of collection fibers from the two-layered phantom. The abbreviation “c.u.” refers to the calibrated unit.

Fig. 7.7 shows fluorescence spectra measured using the system shown in Fig. 7.1. Each spectrum was measured from a different ring of fibers, labeled by the ring number from 1 to 5, with an increasing distance from the assembly’s centre as shown in Table 7.1. FAD peak intensity that represents the fluorescence contribution from top layer increases from Ring 1, which corresponds to the sample surface at 0 mm, to the maximum in Ring 2, which corresponds to a targeted depth of 1.16 mm beneath the surface. FAD peak intensity then goes down from Ring 3 to Ring 4 and reaches the minimum in Ring 5. PpIX peak intensity that represents the fluorescence contribution from the bottom layer increases significantly from Ring 1 to Ring 2 and reaches the maximum in Ring 3 which corresponds to a targeted depth of 2.32 mm. Then, PpIX peak intensity starts to drop from Ring 4 to Ring 5. In both representative peak locations of FAD and PpIX, the peak intensity increase to the maxima

with an increasing targeted depth and then decrease after that. The same trend has been observed and discussed in our previous work presented in Chapter 6. FAD and PpIX peak intensities reach their maxima in different rings of collection fibers, which show that this system is capable of performing simultaneous depth sensitive fluorescence measurements from a range of targeted depths with no mechanical moving components and without altering the lens-sample distance.

To evaluate the performance of this system in depth sensitive measurements, we computed the sensitivities in fluorescence measurements to the top and bottom layers of this system as described. First, the raw spectra measured by the Ring 1 and Ring 2 were multiply by a ratio of 2, as the number of fibers in each of these rings was only half of the number of fibers in the outer three rings, so that the fluorescence intensities measured by all rings had comparable magnitudes. Then, the background subtraction was performed on each spectrum. After that, the FAD peak intensity measured by each ring was divided by the maximum of these peak intensities to get the normalized FAD intensities. Similarly, the PpIX peak intensity measured by each ring was divided by the maximum among these peak intensities to obtain the normalized PpIX intensities. The sensitivity of fluorescence measurements to the top layer was then computed by taking the ratio of the normalized FAD intensity measured by each ring to the sum of the normalized FAD and PpIX intensities measured by the same ring. The sensitivity of fluorescence measurements to the bottom layer was calculated by taking the ratio of the normalized PpIX intensity measured by each ring to the sum of the normalized FAD and PpIX intensities measured by the same ring.

Figure 7.8 Sensitivity of each collection ring to the top (FAD) and bottom (PpIX) layers of the tissue phantom. Collection ring 1 refers to the ring of fibers with the smallest radial distance from the centre and collection ring 5 refers to the ring of fibers with the largest radial distance from the centre of the fiber assembly.

Fig. 7.8 shows the sensitivity of fluorescence measurements achieved by each collection ring in this system to the top and bottom layer of the tissue phantom. It can be seen that the sensitivity to the top layer (FAD) for collection ring 1 was the highest at 0.59, followed by collection ring 2 at 0.56, collection ring 3 at 0.48, collection ring 4 at 0.42 and the lowest by collection ring 5 at 0.37. On the other hand, the sensitivity to the bottom layer for collection ring 1 was the lowest at 0.41, followed by collection ring 2 at 0.44, collection ring 3 at 0.52, collection ring 4 at 0.58 and the maximum by collection ring 5 at 0.63. Compared to the top layer sensitivities for our previous axicon lenses based setup ranging from 0.36 to 0.53, which correspond to a targeted depth varying from 0 mm to 4.6 mm, the top layer sensitivities for the improved setup varied from 0.37 (Ring 5) to 0.59 (Ring 1) for the same range in the targeted depth in this study. The sensitivities to the top layer at a targeted depth of 4.6 mm in both setups were similar but the maximum sensitivity to the top layer in the improved setup increased by approximately 11.3%. Consequently, the bottom layer sensitivities of the previous setup at the targeted depth of 0 mm and 4.6 mm were 0.47 and 0.64 while those of the improved setup in this study were 0.41 and 0.63. This observation indicated the

achievement of a larger range of sensitivity to the bottom layer by the incorporation of collection fibers in multiple rings to the detection configuration.

A cone shell detection configuration has been implemented by the incorporation of circular arrays of collection fibers to the setup. In our previous axicon lenses based setup, we have introduced a cone shell illumination configuration that reduces the volume of shallower layer that were illuminated by the excitation light when measuring from a deep layer. Fluorescence signals in the previous system were collected by the axicon in a cone detection configuration. With the formation of an elongated focal line of 1.7 mm in the axial dimension within a turbid medium, fluorescence signals excited from the enlarged illuminated volume will have higher chance to be collected by the cone detection configuration. In the current setup, the incorporation of five circular arrays of collection fibers at the detection end has introduced five cone shell detection configuration with different ring diameter on the collection axicon lens. When the sample is illuminated by a cone configuration, only the fluorescence signals excited from the corresponding targeted depth will be collected by each collection ring thus improving the depth sensitiveness of this system.

The improvement in the maximum sensitivity to the top layer in the improved setup compared to the previous setup could be attributed to the fact that the collection fibers in Ring 1 shown the inner ring in Fig. 7.3 served as a photon gate that blocks photons not originated from the targeted depth due to its limited numerical aperture. It is speculated that those photons reducing the sensitivity to the top layer mostly came from the deep layer through the tip of the axicon lens, since it is known that the tip of an axicon lens is rounded rather than sharply polished. A similar gating mechanism did not exist in our previous setup thus a portion of those photons from the deep layer can be detected and cause degradation in the sensitivity to the top layer.

7.4 Conclusion

In this study, we improved the spectra acquisition speed of an axicon lenses based setup previously developed for depth sensitive optical measurements by incorporating five rings of signal collection fibers into the detection configuration, which enables the collection of optical spectra from five different depths simultaneously. The improved setup got rid of the mechanical moving part consisting of two axicon lenses that are required to achieve depth sensitive measurements. The improved setup detected fluorescence with a higher sensitivity to the top layer and achieves larger range of sensitivity to both the top and bottom layer in a two-layered turbid phantom. Inheriting the advantage of the previous version of the setup, that is without the need of altering the lens-sample distance in performing depth sensitive measurements, this current setup has enhanced depth sensitivity capability and improved acquisition speed. Hence, this new setup would offer greater convenience and better diagnostic performance in clinical measurements.

Chapter 8 Multifocal Noncontact Color Imaging for Depth Sensitive Fluorescence Measurements of Epithelial Cancer

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8.1 Introduction

Depth sensitive optical spectroscopy has attracted an increasing interest for the diagnosis of epithelial cancers in the past years^{39, 40, 152}. Because the distribution of molecules such as endogenous fluorophores in epithelial tissues is depth dependent and varies significantly with disease stage⁴¹, depth sensitive optical measurements may yield higher sensitivity to malignant growth in epithelial tissues than common optical measurements in which optical signals are averaged throughout the volume being interrogated. A common setup for optical spectroscopy uses a fiber-optic probe for the delivery of illuminating light and collection of emitted light^{153, 154}. In a fiber-optic spectroscopy setup, it is possible to achieve depth sensitive measurements by varying the source-detector separation³⁹, effective aperture diameter of fibers⁴⁰ and illumination and collection angles⁴¹. However, the uncertainty in measurements due to inconsistent probe-sample pressure could induce significant distortion in measured spectra, which consequently would cause large errors in diagnosis¹⁵⁵. Lens based setups have been investigated to perform noncontact optical measurements to overcome this problem. Andree et al.¹⁵⁶ performed spatially resolved diffuse reflectance measurements without physically contacting a tissue sample by using a noncontact setup, which involved a spherical and a flat folding mirror for illumination while two achromatic lenses for detection. Bish et al.¹³⁰ achieved noncontact diffuse reflectance measurements on tissue phantoms and human skin by a lens based noncontact probe. Mazurenka et al.¹³¹ pursued time-resolved diffuse reflectance measurements by a noncontact lens based setup, in which laser scanning was used to achieve imaging. Although these reports addressed the problem of inconsistent

probe-sample contact, none of them are suitable for depth sensitive measurements because of the lack of change in the depth of light focus. To develop a noncontact lens-based probe with excellent depth sensitivity in turbid medium has therefore become the main driving force of this project.

In previous chapters, we have investigated and demonstrated that a cone shell illumination and detection configuration exhibits enhanced depth sensitivity than a conventional cone configuration in a turbid medium. A depth sensitive non contact handheld probe has been developed to implement the novel cone shell configuration and the need to contact with the skin as well as the need to vary the probe-sample distance in targeting different depth is successfully eliminated, making the probe a potential diagnostic tool that offers greater conveniences and consistency in various clinical setting. To improve the speed of acquisition of the probe, a special collection fiber assembly consisting of 5 circular collection fiber arrays has been engineered and incorporated to the probe. The collection fiber assembly has enabled the probe to acquire five depth sensitive spectra simultaneously from five different targeted depths on the same vertical axis in one measurement. The incorporation of this custom designed fiber assembly has effectively improved the speed of acquisition by five times and enhanced the measurement consistency by eliminating all mechanical moving components from the probe. As each of the collection fiber rings has limited numerical aperture and serves as a photon gate that reject photons not originated from the targeted depth, the depth sensitivity of the probe has also improved significantly, driving the probe further towards a solid diagnostic tool to the aid of doctors in various clinical settings.

The handheld probe we developed was designed for point measurements. The advantages of a point measurement are excellent spatial resolution, better position accuracy with more focusing power which results in better signal to noise ratio. However, the main setback of a point measurement system is the slow data acquisition when optical imaging in a

large field of view is desired. Although the incorporation of collection fiber assembly to the handheld probe as discussed in Chapter 7 has enable multiple point measurements, which is equivalent to a 2D line scanning in the vertical axis, the speed of acquisition can still be an issue in clinical settings where a large field of view is usually required. This is especially true in the diagnosis of early epithelial cancer where spatial context of a large skin area in optical images may provide important information for clinical diagnosis. In order to expand our depth sensitive optical probe from a point measurement system to an imaging system with larger field of view, we demonstrate a multifocal noncontact setup to perform depth sensitive fluorescence imaging on early epithelial cancer model. Furthermore, similar to our previous setup, the multifocal setup does not require the mechanical movement of any optical components or sample to achieve depth sensitive optical measurements and thus would be convenient in the clinical setting.

8.2 Experimental Setup and Sample Preparation

Figure 8.1 Schematic of the multifocal noncontact imaging setup. Solid lines with arrows represent excitation light flow while green dotted lines represent emission light flow. Light

propagation is illustrated for one microlens only in the region below the microlens array for clarity.

The schematic of our proposed setup is shown in Fig. 8.1. In the illumination module, a 405-nm laser (iFlex-2000, Point Source Ltd., Hamble, UK) with a maximum output power of 20 mW was used as the excitation source. A beam expander was used to expand the excitation laser beam to achieve a beam diameter of 4 mm before passing through a dichroic mirror. A microlens array (Customized model, Wuxi Opton Tech Ltd, Jiang Su, China) was placed in the optical path after the dichroic mirror to form a multifocal illumination plane. The multifocal illumination plane was then imaged onto a tissue sample by using an electrically focus-tunable lens (HR EL-10-30, Optotune, Dietikon, Switzerland). A microlens array refers to a two-dimensional array of multiple microlenses fabricated on a base substrate. The microlens array in our setup contained around 20×20 micro lenses and each single microlens had a diameter of 250 μm and a focal length of 1000 μm . The filling factor or the ratio of the active refractive area to the entire area of the microlens array was around 75%. A tunable lens refers to an optical lens where its focal length can be varied either mechanically or electrically. In our experiment, an electrically focus-tunable lens was used such that the focal length of the tunable lens can be varied precisely by changing the current applied to it. When the focal length of the tunable lens was changed, we are able to image the multifocal illumination plane at different position on the shadow-side of the tunable lens. Therefore, the depth of focal plane of the microlens array under the sample surface, which will be named as the focal depth in the rest of the paper, can be varied accordingly. By opting an electrically focus-tunable lens, the focal depth inside the sample can be varied easily and precisely without any mechanical movement of the imaging lens or the sample.

In the detection module, photons emerged from the sample was first imaged onto the microlens array's focal plane by the tunable lens and defocused by the microlens array. Then

the photons were deflected by the dichroic mirror towards a long pass filter to block the excitation light, before being focused by a convex lens onto a color camera (AT-200 GE, JAI, San Jose, California, USA) equipped with 3-CCD, which captured images in Red, Green and Blue channels. The distance between the focal plane of the microlens array and tunable lens, labeled as u in Fig. 8.1, was fixed at 5.5 cm; while the distance between the sample surface and tunable lens, labeled as d in Fig. 8.1, was set to 4.5 cm. The focal length of the tunable lens was varied from 2.45 cm to 2.60 cm with an increment around 0.25 mm. Consequently, it can be calculated that the focal depth was varied from -0.8 mm to 5.2 mm approximately with an increment of 0.8 mm assuming that the sample surface corresponded to a focal depth of zero. It should be noted that the filling factor of the microlens array we used is only 75%, which means that a considerable portion of excitation light passing through the array would stay parallel. It was necessary to reduce this portion of excitation light hitting the tunable lens; otherwise it would form a strong focal spot after passing through the tunable lens and serve as the background in the subsequent depth sensitive measurements. In order to solve this problem, a blocker made of aluminum foil with a diameter of around 4.0 mm was placed immediately above the tunable lens to block the parallel light. The distance between the microlens array and tunable lens in our setup was large enough so that the portion of light focused by the micro lenses formed a light beam with a size of 10 mm in diameter on the top surface of the tunable lens, while the portion of parallel light remained 4 mm in diameter on the same surface. This ensured that all parallel light has been effectively blocked. It should be noted that a small amount of focused light that passes through the centre of the microlens array was also blocked, but major portion of the focused light passing through was sufficient for fluorescence imaging.

The setup was evaluated on a two-layered agar tissue phantom. The phantom was prepared based on the recipe and procedure published in earlier work¹²³ with optical properties

mimicking human squamous cervical tissue¹⁴². The thickness of top layer, representing the epithelium, was made to be 500 μm and the thickness of bottom layer, representing the stroma, was made to be 10 mm. The lateral dimension of both layers was made larger than 10 mm to represent a semi-infinite medium. Protoporphyrin IX (PpIX) was added into the top layer at a concentration of 71.1 μM while flavin adenine dinucleotide (FAD) was added into the bottom layer at a concentration of 25.5 μM . PpIX and FAD were chosen in this study as they are endogenous fluorophores that can be found naturally in the skin that fluoresce differently under 405 nm excitation light. PpIX fluoresce in red and FAD fluoresce in green and their non-overlapping peak emission wavelengths at 670 nm and 530 nm, respectively can facilitate the discrimination of fluorescence signals originated from each layer in this study. The concentrations of both fluorophores were chosen so that the magnitudes of both emission peaks intensity fall within the same order. Polystyrene spheres (07310, Polysciences, Warrington, PA,USA) and Nigrosin (N4754, Sigma-Aldrich, St. Louis, MO,USA) were added into each layer at a different concentration to mimic the light scattering and absorption properties of the epithelium and stroma in cervical tissues at 530 nm¹⁵⁷. A layer of plastic wrap was used to separate the top and bottom layer to prevent the diffusion of fluorophores and nigrosin molecules from crossing the interface between the two layers. Scattering coefficients of the tissue phantom (μ_s) at excitation wavelength (405 nm) and at PpIX peak emission wavelength (670 nm) were estimated using Mie theory for the concentration of polysphere used in our experiment. The absorption coefficients of the tissue phantom (μ_a) at both wavelengths were measured for the concentration of Nigrosin used in our experiment. The optical properties of the top and bottom layers in the tissue phantom at the excitation wavelength and the peak emission wavelengths of FAD and PpIX are listed in Table 8.1.

Table 8.1 Optical properties of tissue phantom¹⁵⁷. μ_a refers to absorption coefficient in cm^{-1} ; μ_s refers to scattering coefficient in cm^{-1} .

	405 nm (Excitation)		530 nm (FAD)		670 nm (PpIX)	
	μ_a	μ_s	μ_a	μ_s	μ_a	μ_s
Top layer	1.2,	39.7	1.9,	34.2	1.8,	23.1
Bottom layer	1.4,	250.2	2.2,	215.1	2.1,	174.0

All experiments were performed in a dark room. The focal length of the tunable lens was controlled precisely by changing the current applied via the software provided by the manufacturer. Eight color images were acquired within the range of tunable lens' focal length by increasing the applied current with equal increment. The acquisition time for each measurement was one second. The laser shutter was closed for ten seconds between each measurement to allow fluorescence recovery in case of the possible photo bleaching. The acquired color images were processed using ImageJ software first to pick up the regions of interest and then further processed by a custom programmed image processing code written in Matlab (R2010, MathWorks, USA).

8.3 Results and Discussions

Figure 8.2(a) Color images acquired at different focal depths; (b) Raw RGB values averaged for bright spots at a range of focal depths. The scale bar is 400 μm .

A sequence of color images with different focal depths are shown in Fig. 8.2(a). In each subfigure of Fig. 8.2(a), every circular bright spot refers to one focal spot imaged from the surface or the inside of the tissue phantom. When the focal depth was varied from 0 (i.e. tissue surface) to 4.3 mm, slight color changes can be observed. As the light focus moved deeper, the bright spots gradually varied from faint red to faint green. This trend was more obvious in the color values as shown in Fig. 8.2(b), in which the color values in nine different focal regions were averaged to calculate the mean and standard deviation. It can be seen clearly that when the focal depth was increased from -0.8 mm to 5 mm, both R and G values increased to their peak first and then decreased. However R value (mainly from PpIX in the top layer) reached the peak earlier than G value (mainly from FAD in the bottom layer). Moreover, R value decreased faster than G value. This observation directly demonstrated that our setup was capable of discriminating fluorescence from various depths with different sensitivities, which is essential in depth sensitive fluorescence imaging. B value was relatively low and changed very little with the focal depth as compared to R and G values (much smaller than R and G values with the focal depth). This was due to the fact that the two selected fluorophores in this report contributed little to the blue channel and most blue light was blocked by the long pass filter.

Figure 8.3 Percentage of R and G values as a function of focal depth

To characterize the depth sensitivity of the new setup, the percentage of each of R, G and B values relative to the summation of all was calculated as shown in Fig. 8.3. Since B value changed very little, only the percentages of R and G are shown. Fig. 8.3 shows that the percentage of R value increased a little bit when the focal plane was moved from air to tissue surface, then decreased all the way when the focal plane went deeper from the tissue surface to bottom layer. In contrast, the percentage of G has opposite trend. The trends of these ratios explained why the color of images in Fig. 8.2(a) changed from faint red to faint green when the focal depth increased. The percentage change as a function of focal depth was mainly affected by the numerical aperture of the microlens array, which was 0.14 in our setup. Each single microlens can be treated as an objective lens. It is well known that an objective lens with larger numerical aperture would possess a stronger focusing power thus such a lens would yield a better spatial resolution. Similarly a microlens array with a larger numerical aperture should yield better depth sensitivity because of the stronger focusing power. As a matter of fact, we also tried another microlens array with a smaller numerical aperture from Thorlabs (Product ID:MLA150-5C) and found that this microlens array has much smaller depth sensitivity(results not shown in this report) which demonstrated that the percentage change could be much greater if the microlens array with a considerably larger numerical aperture was used.

Figure 8.4 Schematic of the multifocal noncontact spectroscopy setup. Solid lines with arrows represent excitation light flow while green dotted lines represent emission light flow.

To clarify the source of color changes shown in the Fig. 8.2, fluorescence spectra were measured from the same phantom using a setup similar to that in Fig. 8.1. The setup was modified as shown in Fig. 8.4 in which the fluorescence signals that passed through the long pass filter were focused onto the tip of a collection fiber, with a core diameter of 400 μm and an NA of 0.22, which was coupled to a spectrometer (QE 65 Pro, Ocean Optics, Dunedin, Florida, USA). Apart from that, a 100- μm pinhole was placed above the microlens array to select only one microlens for illumination and detection. The exposure time used for each measurement was two seconds, and a total of eight spectra were acquired.

Figure 8.5 Fluorescence spectra for a range of focal depths, the legend shows the values of focal depths

Detected fluorescence spectra for the same range of focal depths as in Fig. 8.3 are shown in Fig. 8.5. It is interesting to see that PpIX emission peak around 670 nm and FAD emission peak around 530 nm both increased to their maximum first and then decreased when the focal depth increased. PpIX emission peak reached its maximum when the focal depth was 0.80 mm while FAD emission peak reached its maximum later when the focal depth was 1.68 mm. The change in the intensity of PpIX peak was more dramatic than that of FAD peak, likely because the PpIX was in the top layer. It is straightforward to see that the trend in the changes of fluorescence peaks in Fig. 8.5 agreed very well with those of color values as shown in Fig. 8.2(b).

Results shown in Fig. 8.2(a) demonstrated that our setup was able to acquire multifocal color images rapidly. Moreover, Fig. 8.2(b) and Fig. 8.3 showed that depth sensitive optical imaging could be achieved in our setup by varying the focal distance of tunable lens and in turn the depth of multiple focal planes in the sample. Color values of tissues could be used directly for cancer diagnosis¹⁵⁸ or to reconstruct the full spectrum using published algorithms^{159, 160}. Therefore our setup could potentially achieve depth sensitive spectral imaging rapidly that would be faster and cheaper than the traditional spectral imaging

setup. Currently, most spectral imaging setups utilize uniform illumination¹⁶¹, which induces no changes in depth sensitivity thus our setup would offer the advantage of variable depth sensitivity for epithelial cancer diagnosis. Another advantage of our proposed setup is the lower requirement on the excitation laser power because the excitation power is focused only on a finite number of focal spots rather than the entire tissue area.

Currently the field of view of our setup was around 4 mm in diameter, which was limited by the size of microlens array and tunable lens we used. In the 4-mm field of view, totally around 10×10 microlenses were covered, which corresponded to a spatial resolution of around 0.4 mm. Note that the spatial resolution could be easily improved by changing the distances u and d in Fig. 8.1 or using a microlens array with smaller individual microlenses.

Another potential issue in this setup worth discussing is the variation in the focal length of tunable lens with light wavelength, i.e. chromatic aberration. By using the law for the approximation of focal length for thick lenses¹⁶², it was found that the focal length of the tunable lens changed 4% when the wavelength was varied from 405 nm to 670 nm. In our fluorescence imaging setup, chromatic aberration should not be an issue because all fluorescence light was excited by the same laser beam with a single excitation wavelength. The variation in the focal length of tunable lens with wavelength would only result in difference in the detection efficiency in a range of emission wavelengths but not the depth of the excitation light focus. However, chromatic aberration cannot be ignored in a diffuse reflectance imaging setup since the illumination light would also contain a range of wavelengths.

8.4 Conclusion

In summary, we proposed a multifocal noncontact setup capable of performing depth sensitive fluorescence imaging on a two-layered epithelial tissue model. The combination of a microlens array and a tunable lens enables the depth of multifocal plane to be conveniently

adjusted without any mechanical movement of the imaging lens or sample. This advantage is particularly desirable in the clinical setting. Results from the phantom study demonstrated that the setup can achieve depth sensitive color imaging, which was further confirmed by spectral measurements. Color values could be also used to reconstruct full spectra using the previous algorithm for rapid spectral imaging.

Chapter 9 Conclusions and Future Directions

9.1 Conclusions

This dissertation presents a series of studies on the development of non-contact lens based optical probe designs and data analysis methods for depth sensitive optical measurements, particularly Raman and fluorescence measurements, in skin pigmentary disorders. Monte Carlo modeling was explored as a light transport model in turbid layered media for the optimization of our probe designs. Novel illumination and detection configuration with enhanced depth sensitivity compared to conventional optical system such as the microscope was introduced and incorporated into our probe design. The main findings of these studies are concluded as follows.

First, Biochemical Component Analysis (BCA) is a versatile method to decompose and fully utilize the entire Raman spectra in elucidating the biochemical basis of Raman spectra measured from biological samples. This method can be used for the identification of tissue malignancy and the evaluation of treatment outcome in various clinical applications including pigmentary disorders. The method was validated in a cell death model study, in Chapter 3, using K562 cell lines in which cell spectra were decomposed into the contributions from several basic biochemical components including protein, lipid, nucleic acid and glycogen by employing a least squares regression algorithm to quantify the changes of cellular constituents in each death mode. Cell death classification accuracies based on features extracted from BCA were excellent as those achieved based on the features extracted from Principal Component Analysis (PCA) method. Having similar cell death modes classification performance with PCA method, BCA method could provide insight into the fine biochemical changes in cell spectra which will help develop Raman spectroscopy

techniques to monitor cellular changes in various biomedical researches as well as potential medical diagnosis tool for various diseases at the tissue level.

Second, the progressive estimation method is a useful method to improve the depth sensitivity of commercial optical systems which employ cone illumination and detection configuration. Conventional cone illumination and detection systems have limited sensitivity to subsurface measurements due to the dominance of signals originated from overlaying layers. In Chapter 4, we introduced the progressive estimation method which can be used to estimate the contribution of signals from different thicknesses of overlaying layers when performing deep measurements from turbid media. Layer specific optical signals can be obtained easily by subtracting the estimated signal contribution from all shallower layers from the measured signals. As this method involves no physical modification to optical configuration and requires only two acquisitions to produce the targeted layer specific optical signals, it is conveniently and readily being employed and improves the depth sensitivity of any commercial optical system.

Third, Monte Carlo modeling is a powerful tool that can simulate any sets of optical properties in various tissue structures under different light illumination and detection geometries. In Chapter 5, we have employed this method to investigate the depth sensitivity achieved by various combinations of illumination and detection configurations including both cone and cone shell configurations implemented by convex lenses and axicon lenses. Phantom experiments have been carried out to validate Monte Carlo modeling of fluorescence in a two-layered epithelial tissue model. The simulated results can be compared to the experimental results and the Monte Carlo modelling method is able to reproduce the enhanced depth sensitivity of a cone shell configuration as observed in phantom experiments. The development and

validation of this Monte Carlo code provides a fast, inexpensive, reliable and robust computational platform that can assist the planning and optimization of optical designs involving the various illumination and detection geometries prior to the physical development of an optical system for real experiments.

Fourth, the novel cone shell illumination and detection configuration was implemented in an axicon lens-based noncontact handheld probe design to extract depth sensitive optical signals from layered turbid media. The major advantage of the probe is that no alteration in the probe-sample distance is required to perform depth measurements, which will effectively minimize inconsistent optical coupling in most other depth sensitive setups. The probe was evaluated systematically in a fluorescence study and it shows higher sensitivity to bottom layer and larger range of sensitivity to both top and bottom layers than a conventional microscope in a two layered turbid phantom. In Chapter 7, a novel fiber assembly design was incorporated to the probe to improve its acquisition by five times in which optical spectra from five different depths can be collected simultaneously in a single measurement. While the excellent depth sensitivity of the probe is maintained, the new setup gets rid of the mechanical moving part including two axicon lenses that are originally required to achieve depth sensitive measurements, which will further improve the consistency of optical coupling and facilitate clinical uses.

Finally, a multifocal noncontact setup was developed to perform depth sensitive fluorescence imaging on a two-layered epithelial tissue model. This setup expands our depth sensitive optical probe from a point measurement system to an imaging system with larger field of view. The combination of a microlens array and a tunable lens enables the depth of multifocal plane to be conveniently adjusted without any mechanical movement of the imaging lens or sample. The performance of the

setup was evaluated on a two-layered turbid tissue phantom in a fluorescence imaging study and the results were further confirmed by spectral measurements.

9.2 Future Directions

The progressive estimation method described in Chapter 4 was developed for optically homogenous tissue phantom where the scattering coefficients of all layers are identical. However, human skin tissues are optically heterogeneous in nature as the optical properties differ between different tissue layers or types such as epidermis and dermis, normal tissue and disease or tumor and etc. More realistic tissue phantoms which mimic the heterogeneity of human skin tissues should be designed to generate sets of scaling factors which are more useful to extract depth sensitive optical signals from subsurface tissue layers in real clinical application. Besides that, the effect of light absorption was overlooked in the study as described in Chapter 4. Excitation light as well as Raman/fluorescence light will be attenuated due to the light absorption property of the phantom medium. The intensity of excitation light which travels deeper into the phantom and the Raman/fluorescence light which reemits from the surface will be affected by light absorption. The effect of absorption coefficients of the phantom media should also be investigated and accounted for in the progressive estimation method to recover the original intensity of the attenuated layer specific optical signals from the targeted depth. Further works need to be done to refine and improve the progressive estimation method to account for both issues discussed above. An *ex vivo* skin tissue sample study would be ideal for testing the effectiveness of the method after the phantom experiments are completed.

The handheld probe described in Chapter 6 and 7 was designed to address several shortcomings of the current optical system; (1) limited sensitivity to subsurface optical signals in a turbid medium, (2) the pressure exerted on the tissue sample by fiber-optic based probe during measurements which causes distortion to measured spectra, and (3) the need to alter probe-sample distance for noncontact lens-based handheld probe which introduces inconsistency to optical measurements. An axicon lens-based handheld probe with a novel cone shell illumination and detection configuration was demonstrated to be able to address the three limitations described above. The incorporation of a custom design collection fiber assembly which consists of five circular arrays of collection fibers has further improved the acquisition speed of the probe by enabling optical spectra to be collected from five different depths in a single measurement. The performance of the probe has been tested systematically in fluorescence study using a two-layered tissue phantom. In the future, the probe will be tested for its clinical efficacy in a real human skin tissue for the diagnosis of skin pigmented disorders.

The Raman spectra of basic components in human skin will be measured by a commercial Raman spectroscope (Invia, Renishaw, New Mills, UK) to be used as basis spectra in the regression. These components include filaggrin and keratin, which are the main structural proteins found in epidermal keratinocytes, melanin, which is produced by melanocytes located at the bottom layer of the skin's epidermis or stratum basale, collagen, lipid, water and other biochemical components to be included to improve the fitting of the spectrum. The skin from patients with suspected malignant pigmented lesions will be measured by the handheld probe as described in Chapter 7 before the biopsy is taken. The Raman spectra at all depths will be processed using progressive estimation method to derive the contribution of all components at each

depth. Since the overproduction or accumulation of melanin is the main cause of pigmented lesions, the depth of the pigmented lesion can be estimated by plotting the concentration of melanin against the depth of focus and identifying the peak of the curve. The full-width half-maximum (FWHM) of the peak is the estimated size of the pigmented lesions. These values will be validated against the histopathology of tissue biopsies that are performed after Raman measurements. Alteration in the positions, shapes and intensities of spectral bands for protein and lipid are reported to be able to detect the malignancies of the lesions such as the development of melanoma which is the most aggressive skin cancer.

Even though the handheld probe was tested to be effective in phantom experiments, there are several possible challenges when performing real clinical measurements. In the phantom study, the surface of the phantom was made to be flat where the sample surface or zero position relative to the tip of the probe can be controlled precisely. However, in the real clinical situation, a lesion could grow upwards and form a hump, or rough and uneven site on the skin surface which causes difficulty in focusing on the skin surface or the zero position relative to the probe tip. Besides that, in the phantom experiment, semi-infinite tissue models were used where each layer of tissue phantom has finite thickness and clear boundary between layers; however, the size and shape of a pigmented lesion are usually irregular and without clear boundary between surrounding tissue. Furthermore, unlike phantom studies, there is a maximum permissible exposure (MPE) regulation for clinical laser application which limit the laser power density being illuminated on human tissues. Since the Raman scattering cross-sections of biological tissues are inherently small and the measured Raman spectrum is expected to be noisy (low S/N ratio). Thus it is

very important to test the probe in a real clinical study to evaluate its clinical efficacy and improve the system for every challenges encountered.

Author's Publications

Academic Journals

1. Y. H. Ong, M. Lim, and Q. Liu, "Comparison of principal component analysis and biochemical component analysis in Raman spectroscopy for the discrimination of apoptosis and necrosis in K562 leukemia cells," *Optics Express*, Vol. 20 Issue 20, 22158-22171 (2012).
2. Y. H. Ong, and Q. Liu, "Axicon lens-based cone shell configuration for depth-sensitive fluorescence measurements in turbid media," *Optics Letters*, Vol. 38 Issue 15, 2647-2649 (2013)
3. Y. H. Ong, and Q. Liu, "Fast depth-sensitive fluorescence measurements in turbid media using cone shell configuration," *Journal of Biomedical Optics*, Vol. 18 Issue 11, 110503 (2013)
4. S. Chen, Y. H. Ong, and Q. Liu, "Fast reconstruction of Raman spectra from narrow-band measurements based on Wiener estimation," *Journal of Raman Spectroscopy*, Vol. 44 Issue 6, 875-881 (2013)
5. C.G. Zhu*, Y.H. Ong*, and Q. Liu, "Multifocal noncontact color imaging for depth-sensitive fluorescence measurements of epithelial cancer," *Optics Letters*, Vol. 39 Issue 11, 3250-3253 (2013) { *equal contribution }
6. Y.H. Ong, C.G. Zhu, and Q. Liu, "Phantom Validation of Monte Carlo Modeling for Non-contact Depth Sensitive Fluorescence Measurements in an Epithelial Tissue Model," *Journal of Biomedical Optics*, Vol.19 Issue 8, 085006 (2014)
7. S. Chen, Y.H. Ong, X.Q. Lin, Q. Liu, "Optimization of advanced Wiener estimation methods for Raman reconstruction from narrow-band measurements in the presence of fluorescence background," *Biomedical Optics Express* (Under review)

Conferences and Presentations

1. **Y. H. Ong** and Q. Liu, "Comparison of cone and cone shell configuration for depth sensitive fluorescence measurements in turbid media," in Proc. SPIE 8926, *Photonic Therapeutics and Diagnostics X*, p. 89260J, San Francisco, California, USA (2014)
2. **Y. H. Ong** and Q. Liu, "Axicon lenses based cone shell configuration for depth sensitive fluorescence measurements in turbid media," *Photonics in Dermatology and Plastic Surgery, The International Society for Optical Engineering (SPIE) International Symposium on Biomedical Optics* in Photonics West 2014, San Francisco, California, February 1, 2014.

3. **Y. H. Ong**, C. Zhu and Q. Liu, "Experimental validation of Monte Carlo modeling of depth sensitive fluorescence illumination and detection configurations in skin tissues," *Optical Biopsy XII, The International Society for Optical Engineering (SPIE) International Symposium on Biomedical Optics* in Photonics West 2014, San Francisco, California, February 4, 2014.
4. S. Chen, **Y. H. Ong**, X. Q. Lin, R. Chen and Q. Liu, "Fast reconstruction of Raman spectra from wide-band measurements of Raman signals with fluorescence background," *Optical Biopsy XII, The International Society for Optical Engineering (SPIE) International Symposium on Biomedical Optics* in Photonics West 2014, San Francisco, California, February 4, 2014.
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8. S. Chen, **Y. H. Ong** and Q. Liu, "Fast reconstruction of Raman spectra from narrow-band measurements based on Wiener estimation", *Optics in Health Care and Biomedical Optics V, SPIE Photonics Asia*, Beijing, China, Nov. 7, 2012.
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