Development of Raman spectroscopy for biological sample analysis

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2017

Chen, K. (2017). Development of Raman spectroscopy for biological sample analysis.
Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/72132>

<https://doi.org/10.32657/10356/72132>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**Development of Raman spectroscopy for
biological sample analysis**

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**SCHOOL OF CHEMICAL & BIOMEDICAL
ENGINEERING**

2017

Development of Raman spectroscopy for biological sample analysis

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School of Chemical & Biomedical Engineering

A Thesis Submitted to the Nanyang Technological University

In fulfillment of the requirement for the degree of

Doctor of Philosophy

Jan 2017

Development of Raman spectroscopy for biological sample analysis

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Under the supervision of Assistant Professor Liu Quan

At Nanyang Technological University, Singapore

Acknowledgements

I would like to express my utmost thanks to everyone that has helped me during my PhD study. First of all, I would like to thank my supervisor, Dr. Liu Quan, for his great effort in supporting me during the past four years. His expertise and experiences has benefited me significantly in my PhD study. He always inspires me and offers me many insightful suggestions whenever I encountered difficulties in my research. He was always my primary choice when I need any help during PhD study. Besides, I also want to thank those professors and staffs who taught or helped me at NTU for their valuable advices on either research or career, especially those serving in my qualifying examination and PhD defense committees. Moreover, I would like to thank my friendly group members for their help and support during these years. Without them, my PhD life would be less joyful. Finally, I would like to thank my family, my wife and my friends for their continuous support.

Table of Contents

List of Figure Captions	3
List of Table Captions	5
Abstract	6
Chapter 1: Overview	9
Chapter 2: Introduction	12
2.1 Principle of Spontaneous Raman Spectroscopy.....	12
2.2 Principle of Surface Enhanced Raman Spectroscopy (SERS).....	13
2.3 Neural Stem Cell (NSC) Differentiation and Techniques for NSC Isolation	17
2.3.1 <i>NSC differentiation</i>	17
2.3.2 <i>Neurological disease and stem cell therapy</i>	17
2.3.3 <i>Raman spectroscopy for stem cell measurement</i>	19
2.4 Corneal Infection Diagnostics.....	21
2.4.1 <i>Corneal infection</i>	21
2.4.2 <i>Current diagnostic techniques</i>	22
2.5 Malaria Infection Diagnostics	23
2.5.1 <i>Status of Global Malaria Infection and its Life Cycle</i>	23
2.5.2 <i>State of Art Techniques for Malaria Diagnosis</i>	24
Chapter 3: Raman Spectroscopy for Differentiation between Neural Progenitor Cell and its Lineages	28
3.1 Raman Spectroscopy in Neural Stem Cell Detection	28
3.2 Methodology	29
3.2.1 <i>Cell culture and sample preparation</i>	29
3.2.2 <i>Raman measurements and data processing</i>	31
3.3 Results.....	33
3.4 Discussion	38
3.5 Conclusion	41
Chapter 4: Raman Spectroscopy for Corneal Infection Diagnosis	42
4.1 Introduction.....	42
4.1.1 <i>Raman spectroscopy for fungi detection</i>	42
4.1.2 <i>Raman spectroscopy for cornea measurement</i>	43
4.2 Methodology	44
4.2.1 <i>Fungi culture</i>	44
4.2.2 <i>Raman measurements and data processing</i>	46
4.3 Results.....	48
4.4 Discussion and Conclusion	56
Chapter 5: Surfaced Enhanced Raman Spectroscopy for Early Malaria Diagnosis	60
5.1 Raman Spectroscopy (RS) and SERS in Malaria Research and Diagnosis...	60
5.1.1 <i>Qualitative Raman Measurements</i>	60
5.1.2 <i>Quantitative Raman and SERS Measurements</i>	62
5.2 Methodology	63
5.2.1 <i>Parasite culture</i>	63

5.2.2	<i>Sample preparation in Method 1 (synthesizing nanoparticles separately).....</i>	64
5.2.3	<i>Sample preparation in Method 2 (synthesizing nanoparticles inside parasites).....</i>	65
5.2.4	<i>Raman measurements and data processing</i>	66
5.2.5	<i>Detection limit estimation</i>	68
5.2.6	<i>Ethics statement</i>	68
5.3	Results.....	68
5.4	Discussion	74
5.5	Conclusion	83
Chapter 6:	Surface Enhanced Raman Spectroscopy for Single Parasite Detection.....	84
6.1	Raman Spectroscopy and SERS in Single Parasites Detection	84
6.2	Methodology	85
6.2.1	<i>Parasite culture</i>	85
6.2.2	<i>Hemozoin isolation</i>	86
6.2.3	<i>Red blood cell lysis</i>	86
6.2.4	<i>Synthesis of silver nanoparticles and preparation before Raman measurements</i>	86
6.2.5	<i>Synthesis of silver nanoparticles (NPs) inside parasites</i>	87
6.2.6	<i>Raman measurements and data processing</i>	88
6.2.7	<i>Procedure of sample postprocessing to confirm the existence of parasites in the ring stage at the focal location.....</i>	90
6.2.8	<i>Ethics statement</i>	90
6.3	Results.....	91
6.4	Discussion	96
6.5	Conclusion	99
Chapter 7:	Conclusions and Future Directions	100
7.1	Conclusions.....	100
7.2	Future Directions	102
Author publications.....		105
Academic Journals		105
Book Chapter and Conferences Volume		105
Conference Presentations.....		106
References.....		108

List of Figure Captions

- Figure 2.1** Comparison of the current techniques for malaria diagnosis in terms of sensitivity.27
- Figure 3.1** Raman spectra of NPC, astrocyte, OL and OPC, respectively, from the bottom curve to the top curve. The shaded region represents the respective standard deviation ($\text{Mean} \pm 1\text{SD}$).33
- Figure 3.2** Plot of the second PC (PC2) and the fourth PC (PC4) from all cell spectra.34
- Figure 3.3** Raman spectra of NPC measured with excitation at 514 nm (blue curve), 633 nm (green curve) and 785 nm (red curve).35
- Figure 3.4** Raman spectra of astrocytes measured using 785 nm excitation with the power varying from 1 mW to 50 mW and the exposure time from 1 second to 10 seconds.37
- Figure 4.1** Slit lamp images of rabbit eyes infected by (a) *Fusarium solani* (F. s.), (b) *Candida albicans* (C. a.) and (c) *Aspergillus fumigatus* (A. f.). The infected regions are marked by black arrows.48
- Figure 4.2** Raman spectra from *Aspergillus fumigatus* (A. f.), *Fusarium solani* (F. s.) and *Candida albicans* (C. a.). Ten spectra were captured and averaged for each species. The shaded region represents the respective standard deviation ($\text{Mean} \pm 1 \bullet \text{SD}$). Note that the spectra were not normalized.49
- Figure 4.3** Raman spectra from healthy rabbit corneas, F. s. infected corneas, C. a. infected corneas and A. f. infected corneas. The shaded region represents the respective standard deviation ($\text{Mean} \pm 1 \bullet \text{SD}$). Note that the spectra were not normalized.50
- Figure 4.4** (a) Plot of the first PC (PC1) score vs. the second PC (PC2) score calculated for all corneas spectra. (b) Plot of the first PC (PC1) score vs. the fourth PC (PC4) score calculated for group 1 (healthy corneas and F. s. infected corneas) and group 2 (C. a. infected corneas and A. f. infected corneas). (c) Plot of the first PC (PC1) score vs. the second PC (PC2) score calculated for healthy corneas and F. s. infected corneas. (d) Plot of the first PC (PC1) score vs. the second PC (PC2) score calculated for C. a. infected corneas and A. f. infected corneas. The black lines in (b), (c) and (d) indicate the boundary between two groups in each figure.53
- Figure 5.1** SERS spectra and hemozoin contribution in Method 1. (a) SERS spectra contributed by hemozoin in infected blood treated by Method 1. (b) Hemozoin contribution as a function of parasitemia level. In (a), the data point corresponding to a parasitemia level of 0.01% is marked by a red box. In (b), the resulting curve of the second-order polynomial fitting for the data points corresponding to parasitemia levels in the range of 0.01% to 0.2% is shown in red. The data corresponding to normal blood samples, labeled as “NB”, is added manually to facilitate comparison. The y axes in both figures are plotted in arbitrary units, labeled as “A. U.”69
- Figure 5.2** SERS spectra and hemozoin Raman peak distribution in Method 2. (a)

SERS spectra of normal blood and infected blood sample treated by Method 2. (b) SERS peak intensity at 1623 cm^{-1} as a function of parasitemia level. The data corresponding to normal blood samples, labeled as “NB”, is added manually to facilitate comparison. The y axes in both figures are plotted in arbitrary units, labeled as “A. U.”.....70

Figure 5.3 Giemsa stained images of blood samples treated by Method 1 and Method 2. (a) a blood sample with parasites in the ring stage prior to cell lysis, (b) silver nanoparticles alone, (c) a blood sample after blood cell membrane lysis but prior to nanoparticle synthesis treated by Method 2, (d) the blood sample after nanoparticle synthesis treated by Method 2 and (e) a blood sample treated by Method 1 and then mixed with nanoparticles synthesized separately.....72

Figure 5.4 FESEM image of silver nanoparticles synthesized in Method 1. The nanoparticle size ranges from 50 to 100 nm.74

Figure 6.1 (a) Spontaneous Raman spectra of hemozoin isolated from infected blood samples, infected RBCs in the schizont stage, infected RBCs in the ring stage and normal blood. The original amplitudes of all spectra are maintained to facilitate comparison. (b) A white light microscopic image of infected blood that contains infected RBCs in the schizont stage and the ring stage as well as normal RBCs. (c) A Giemsa stained image taken from the same region in (b). An infected RBC in the schizont stage, an infected RBC in the ring stage and a normal RBC are indicated, respectively, by the thin solid arrow (red), thick solid arrow (blue) and thin dashed arrow (green) in (b) and (c).91

Figure 6.2 (a) SERS spectra of hemozoin, parasites (in the ring stage) with silver nanoparticles synthesized inside and parasites (in the ring stage) mixed with nanoparticles. The original amplitudes of all spectra are maintained to facilitate comparison. (b) Giemsa stained image of parasites with nanoparticles synthesized inside from the center of which Raman measurements were taken. (c) Giemsa stained image of parasites mixed with nanoparticles from the center of which Raman measurements were taken. 94

List of Table Captions

Table 3.1 Classification of all cell spectra acquired from NPC, astrocyte, OL and OPC. The reference cell type is also included to indicate the accuracy of classification.	34
Table 4.1 One-step classification of all spectra acquired from healthy corneas, F. s. infected corneas, C. a. infected corneas and A. f. infected corneas.	53
Table 4.2 Step-wise classification of Raman spectra acquired from healthy corneas, F. s. infected corneas, C. a. infected corneas and A. f. infected corneas.....	54
Table 5.1 Sensitivities of current methods in malaria diagnosis. The percent parasitemia level is converted to the number of parasites per microliter with the assumption that normal blood in human body contains 5×10^9 RBCs/ml.	74
Table 5.2 Comparison in the peaks of SERS spectra between Method 1 and Method 2. The peaks of spontaneous Raman spectra measured from hemozoin in parasites' vacuoles in the literature[82] and their assignment are also listed for comparison[128].	78

Abstract

This dissertation presents a series of studies in the development of Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) techniques in biological sample analysis, particularly for the characterization of cells and tissues and biomarker detection.

First, the background in the target applications and currently available methods are introduced. Spontaneous Raman spectroscopy, surface enhanced Raman spectroscopy and the associated methodology are described to establish the context for the development of more advanced methods in the subsequent chapters.

Spontaneous Raman spectroscopy was first applied in the discrimination of neural stem cells and their lineages, to guide the fundamental research for neural stem cell therapy for neurological diseases. Spontaneous Raman spectra were acquired from single cells and then multi-variate analysis was conducted to classify cells and yielded high accuracy. Moreover, we optimized experimental parameters for cell measurements by quantitatively comparing the spectra obtained with various system parameters.

Spontaneous Raman spectroscopy was then applied to sample characterization at the tissue level. The feasibility of using spontaneous Raman spectroscopy to diagnose corneal infection caused by fungi was demonstrated. Moreover, it was possible to determine the species of infectious agent. Characterization was enhanced by measuring fungi spores of different species, in which Raman spectra showed significant differences. By use of multi-variate analysis, infected corneas can be easily distinguished from healthy corneas and high accuracy was achieved in the classification

of fungi species.

To enhance the sensitivity to a particular biomarker for early malaria diagnosis, two methods of SERS were developed to enable the detection of hemozoin, which is a common biomarker for malaria infection, at a range of low concentrations in human blood. In Method 1, silver nanoparticles were simply mixed with preprocessed blood samples; while in Method 2, silver nanoparticles were synthesized inside malaria parasites so that hemozoin would not be diluted prior to measurements. A characteristic SERS peak intensity was used to estimate hemozoin concentration and malaria parasitemia level. The results indicated that Method 2 can achieve ultra-sensitive detection of hemozoin exceeding the sensitivity of the standard method that is based on microscopic examination of Giemsa stained blood smears.

It was then realized that Method 2 in the previous study could be employed for the detection of single parasites due to its high sensitivity. By developing a method that enables the association of measured SERS spectra with stained parasites, we demonstrated for the first time that hemozoin from single parasites in the ring stage can be detected.

In summary, we have developed a series of spontaneous Raman spectroscopy and SERS techniques and demonstrated their applications in biological sample analysis at different levels. In particular, spontaneous Raman spectroscopy was found highly effective in the differentiation of neural stem cells and their lineages and corneal infection diagnosis. In contrast, SERS can perform ultra-sensitive detection of malaria biomarker for early malaria infection diagnosis. These techniques could be adopted in

biological science research and clinical diagnosis once incorporated into a custom built Raman system tailored for particular applications.

Chapter 1: Overview

This dissertation presents a series of studies in the development of Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) in biological analysis, particularly for characterization of single cells and tissues and biomarker detection. The following chapters are arranged in a logical sequence in which the significance of study, the shortcoming of current diagnostic or detective techniques, Raman spectroscopic techniques and our methods are described.

Chapter 2 introduces the target tissue types, i.e. neural stem cells, corneal infection and malaria infection, in which the brief biomedical status and significance are described. In addition, the current diagnostic methods are discussed and current state of Raman spectroscopy in the field is reviewed.

Chapter 3 depicts Raman spectroscopy in the discrimination between neural stem cell and its lineages. Spontaneous Raman spectra were obtained from single cells deposited on aluminum substrate and then principal component analysis combined with linear discriminant analysis (PCA-LDA) was conducted on the Raman spectra to classify them into specific cell type. With high classification accuracy achieved, Raman spectroscopy provides a non-invasive and rapid cell detection and classification method for neural stem cell development, especially in the situation where cell concentration is low.

Chapter 4 describes spontaneous Raman spectroscopy technique for fungi induced corneal infection diagnosis. In this study, Raman spectra were initially obtained from different fungi spores to demonstrate the feasibility of Raman in differentiating fungal

species. Then rabbit corneas infected by varied fungi were illuminated by near infrared laser with a large focus spot and Raman spectra were acquired from the suspicious freckles on cornea surface. The spectra from infected cornea were then compared with those from healthy rabbit cornea and then PCA-LDA method was implemented to classify them to indicate the infectious agent.

Chapter 5 demonstrated two methods of SERS technique for early malaria diagnosis in the ring stage. Method 1 was conducted by mixing silver nanoparticles with isolated biomarker-hemozoin from malaria infected human blood; while in Method 2, silver nanoparticles were synthesized inside malaria parasites. Method 1 shows smaller data variation and better correlation between hemozoin contribution and the parasitemia level, thus more reliable quantitative approach, than Method 2. Whereas, Method 2 achieves ultra-sensitive detection limit in the ring stage, the best sensitivity over current methods, to our best knowledge.

Chapter 6 investigated the existence of hemozoin in single malaria parasites in the ring stage using SERS. We combined the protocol Method 2 described in Chapter 5 with Giemsa stained microscopy. After silver nanoparticles synthesis inside parasites, Raman spectra were acquired from the parasite vacuoles consist of silver nanoparticles. Then the aggregates were stained by Giemsa stain to confirm the existence of parasites. Here we demonstrated for the first time detection of hemozoin in malaria parasites in the ring stage and this will greatly facilitate the fundamental research on malaria development and anti-malaria drug development.

In Chapter 7, we conclude the major findings and novel inventions in this

dissertation and propose the future direction of Raman spectroscopy in biological application.

Chapter 2: Introduction

2.1 Principle of Spontaneous Raman Spectroscopy

Raman spectroscopy (RS) is a photonic scattering process from which chemical information is derivable. A typical Raman Stokes-scattering process involves excitation photons, originating from a monochromatic source, inelastically colliding with the atoms comprising a material. Varying portions of energy from these photons translates into various molecular vibrational modes dependent on the types of atoms and their chemical bonds. The remaining energy, emitted as photons, is detectable by a spectrophotometer to produce a Raman spectrum. To be more general, photons from the excitation source, which is usually a laser, excite a molecule to a higher virtual energy state and then photons with energy different from the excitation photon are emitted, when the molecule returns to the ground state. The series of peaks in a spectrum concertedly reflect the distinctive chemical makeup of a material.

Raman spectroscopy boasts numerous advantages as a detection modality and attracted considerable attention as a tool in characterization research. It is a label free technique that can conveniently probe live cells due to the low scattering ability of water without using extrinsic labels such as molecular markers and stains that may undesirably perturb readings. Some chemicals such as hemozoin crystals serendipitously exhibit strong absorption bands in the near-IR region enabling strong resonance enhancement at various wavenumbers.[1] This is known as resonance Raman spectroscopy (RRS) where enhancement is obtained by coordinating the excitation wavelength with the absorption band of an analyte.

However, spontaneous RS has low sensitivity due to weak Raman scattering intrinsic to most biological materials that hamper the efforts of detection at low concentration. Additionally, Raman enabled microscopy systems are impractical for routine field use.

2.2 Principle of Surface Enhanced Raman Spectroscopy (SERS)

SERS is most widely exploited to dramatically enhance Raman signals by many orders of magnitude. This effect arises when analytes are adsorbed or reside closely to a metal surface, typically gold or silver nanostructures, to benefit from surface plasmons, i.e. oscillating electron clouds. A material's characteristic surface plasmon is size dependent and maximally excited upon exposure to light matching its plasmon frequency to achieve a resonant state.[2] This generates localized electromagnetic (EM) radiation near the surface and causes molecules at close proximity to emit enhanced Raman signals due to the EM field amplified by resonant plasmons.[3] An enhancement factor of up to 10^{14} has been reported where the enhancement is intensified even further when a single molecule was detected.[4] While the EM mechanism accounts for most of the observed enhancement, a secondary mechanism, namely chemical enhancement, may also play a role.[5] This is theorized to involve an alteration of electronics states of the adsorbed analyte to enable resonance Raman scattering.[6] More details about the SERS principle are described as follows.

Electromagnetic enhancement (EME)

When the incident light or an electromagnetic wave interacts with a metal surface,

localized surface plasmons are excited where the magnitude of EM fields at the surface are enhanced. Localized surface plasmon resonance (LSPR) occurs when the frequency of the incident light matches the natural frequency of surface electrons. The field enhancement is the greatest during LSPR when the collective oscillation of these valence electrons in the metal surface is in resonance with the frequency of incident light. Rough surface is required to create a component of the plasmon that oscillates perpendicular to the surface plane for scattering to occur.[7] The electromagnetic enhancement is therefore dependent on the presence of the metal surface's roughness features. Rough metal surfaces and nanoparticles of different shapes, such as nanorod[8], nanocrescent[9], nanotriangle[10], nanoflower[11], nanocube[12], or nanopyramid[13], have been fabricated to provide areas or hotspots on which these localized collective oscillations can occur.

The intensity of the Raman scattered radiation is proportional to the square of the magnitude of the EM field incident on a test molecule, i.e.

$$I_R \propto E^2 \quad (1)$$

where I_R is the intensity of the Raman field and E is the overall EM field coupling with the analyte (summation of the EM field on the analyte in the absence of all roughness features and that emitted from the particulate metal roughness feature).[14, 15] SERS amplification is extremely strong because field enhancement occurs twice. First, LSPR amplifies the intensity of incident light that excites test molecules on the metal surface.

Then, the Raman scattered light experiences enhancement by the same mechanism augmenting the incident light. Thus, the total enhancement experienced by each test molecule is approximately the product of these two events of field enhancements:

$$\text{Enhancement Factor (EF)} \approx |E(\omega)|^2 |E(\omega')|^2 \quad (2)$$

where $E(\omega)$ represents the field enhancement factor experienced by the incident light at a frequency of ω and $E(\omega')$ represents the field enhancement factor experienced by the Raman signal at a Stokes-shifted frequency of ω' . Therefore, the electromagnetic SERS enhancement shows the fourth-power dependency on the EM field amplitude, $|E(\omega')|^4$, when $\omega = \omega'$. [16, 17] This enhancement factor experienced by the test molecule is the strongest when it is in direct contact with the metal surface. For molecules at a distance, d , away from the surface of a metal sphere, the enhancement factor deteriorates by a coefficient of $[r/(r+d)]^{12}$, where r is the radius of the spherical surface. [18, 19] This shows that the average distance of metallic surfaces/nanoparticles introduced to specific cutaneous components of interest will directly affect the overall enhancement factor of intradermal SERS signals. Therefore, the methods of introduction as well as the fabrication of these metallic surfaces/nanoparticles are critical in defining the effectiveness of SERS measurements.

Chemical enhancement (CE)

Chemical enhancement provides one or two order(s) of magnitude enhancement in the

intensity of Raman scattering. Despite the detail of this mechanism is less clear than EM enhancement, it is evident that CE is due to the change in the electronic structure of an analyte molecule when absorbed onto metal surface that enhances its Raman polarizability.[18, 20] When an analyte molecule is adsorbed onto a metal surface, the interaction between the analyte molecule and metal surface may create a pathway of electronic coupling allowing for electron transfer between the orbital of the molecule and that of the metal. The electronic coupling between the metal and the molecule establishes a new equilibrium for the relaxation of the molecule, increasing its Raman scattering cross section.[21, 22] Apart from the formation of charge-transfer intermediates, CE has also been described in several ways of metal-adsorbate interaction, including metal-molecule electron tunneling[23], intensity borrowing[24], and formation of metal-molecular surface states.[25] Despite the difference in their enhancement mechanisms, both EME and CE require a small distance between the metallic surface and the analyte molecule.

Although these mechanisms are not yet proven, there is general agreement that they are responsible for SERS. The ultra-high sensitivity of SERS is the greatest advantage over standard RS. SERS has been used to detect nuclear and cytoplasmic materials within cells as well as proteins at low concentrations.[26]

The performance of SERS is highly related to the interaction between targeted molecule and the surface of plasmonic structures. The most common plasmonic materials used in SERS is gold (Au), silver (Ag) and copper (Cu). Besides, some alkali metals and novel material such as graphene, semiconductor and quantum dots have

been explored to form SERS substrate [27]. In this study, we will use silver nanoparticles, since they are cost effective and easy to synthesize. In addition, silver nanoparticles show resonance vibration in hemozoin detection, which has been demonstrated in the work done by Yuen et al.[28]. The details will be discussed in section 5.1.2.

2.3 Neural Stem Cell (NSC) Differentiation and Techniques for NSC Isolation

2.3.1 NSC differentiation

Neural stem cells are self-renewing, multipotent cells which can differentiate into neurons and glia in the nervous system, mostly in the embryonic stage. Although NSCs can primarily differentiate into neurons, astrocytes and oligodendrocytes (OL) during the embryonic period, only some NSCs can persist to generate neurons in a few restricted regions of adult brain spontaneously[29]. And thus NSC development *in vivo* shows great significance in diagnosis and treatment for neurological diseases.

2.3.2 Neurological disease and stem cell therapy

Neurological diseases refer to the disorders of the brain, spine and the nerves that connect them. Currently, there are more than 600 diseases in the nervous system, such as brain tumors, Parkinson's disease, Alzheimer's disease and stroke. Most of the neurological disorders are originated from the death of neurons or glia which may be caused by injury, infection or cancer.

Neurological diseases are one of the main causes of adult disability and they are

estimated to cause more deaths than cancer in the elderly population by 2040.[30] In consideration of the aging population, it is important to develop effective therapeutic strategies. Much progress has been achieved in the clinical and theoretical area for neurological diseases.[31-36] One major problem to be resolved in the treatment of neurological diseases is that injuries in the nervous system are always irreversible, since the neurons and glia in the adult central nervous system (CNS) are unable to regenerate spontaneously. Due to the potential to overcome the intrinsic inability of CNS to replace damaged elements, stem cell transplantation has attracted much attention. It has been shown that the symptom of Parkinson's diseases can be improved by transplanting fetal mesencephalic cells into patients.[37] Significant treatment of Huntington's diseases was also achieved when patients received fetal striatal grafts. However, even with all the promising results, there are significant constraints to fetal stem cell therapy that limits the usage in the field. Firstly, there is always ethical concern on using aborted fetuses and its viability and purity cannot be optimally controlled. Moreover, fetal cells are difficult to be expanded or stored for even a few days, so the time between surgery and tissue donation always has to be short.

To facilitate the stem cell therapy and replace fetal stem cell in neurological disease treatment, neural stem cells and neural progenitor cells come into the sight. The neural stem cells (NSCs) and neural progenitor cells (NPCs) can be isolated from adult CNS and then be reliably cultured in culture media for a long period. The study of NSCs shows their potential to generate unlimited neurons and glia by cell transplantation. It has been reported in the literature that several neurological diseases benefit from the

NSC therapy.[34, 38] However, the mechanisms how NSCs differentiate in the clinical transplantation are not fully understood. Among all the fundamental research about neural stem cells, one common key technique is required for cell detection and classification. Currently, the standard microscopy based technique focuses on fluorescence staining of a unique biomarker in cells and observe the morphology with a microscope.[39] Although fluorescence microscopy is effective, its major disadvantage is the requirement of cell fixation and fluorescence staining which will definitely kill the cells. Besides, the performance of fluorescence staining relies on the effectiveness of the dye and the operator's skills. Some other commercially available techniques are also applied to NSC detection, such as MRI and PCR.[40, 41] But they both encountered the problems of high cost and intensive labor required.

2.3.3 Raman spectroscopy for stem cell measurement

As a fast, label-free and non-invasive technique, Raman spectroscopy has been applied in stem cell detection decades ago. The vibrational features of Raman spectra have been used to differentiate stem cells from their lineages. Embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs) are most frequently studied types among all as surveyed below.

Notingher *et al.*[42] used Raman spectroscopy to observe nucleic acid changes in murine embryonic stem cells (ESCs) during differentiation. They found that the contribution of DNA and RNA decreased in Raman spectra. Significant differences could be observed in ESCs between undifferentiated and late differentiation (16 days) stages. Besides, they [43] also monitored the mRNA translation in ESCs during

differentiation using the same method. Chan and Lieu[44] compared the Raman spectra from human ESCs, ESCs-derived cardiac myocytes (CMs) and the more mature fetal left ventricular cardiomyocytes (FLV-CMs). Their results also showed that RNA component contributed more in undifferentiated stem cells compared to differentiated cells. Moreover, they demonstrated the sensitivity for discrimination for ESCs, CMs and FLV-CMs were 98%, 96% and 66%, respectively, using the principal component analysis combined with linear discriminant analysis (PCA-LDA). Pascut *et al.*[45] worked on differentiated human ESCs-derived CMs within heterogeneous cell population. The results showed that glycogen bands acted as a main marker in Raman spectra for discrimination. By combining PCA and the cross-validation strategy, they achieved 96% sensitivity and 97% specificity.

Konorov *et al.*[46] utilized Raman spectroscopy to investigate the cytochemical distribution in human ESCs colonies. Their results showed that there were significant differences between pluripotent and differentiated colonies. In addition, Konorov *et al.*[47] also showed the feasibility of quantifying glycogen in hESCs using Raman spectroscopy.

Besides ESCs, Raman spectroscopy showed the capacity to monitor the cellular and molecular development during differentiation of mesenchymal stem cells (MSCs). Azrad *et al.*[48] compared mesenchymal stem cells (MSCs) treated with quality elk velvet antler (QEVA) and those with the osteogenic agent dexamethasone (Dex) using confocal Raman spectroscopy. The results showed that, cell cultures supplemented with QEVA developed more hydroxyapatite than cell cultures treated with Dex. McManus

et al.[49] reported the detection of formation of a bone-like apatite mineral during the differentiation of human MSCs towards an osteogenic lineage using Raman spectroscopy, in which multivariate analysis was performed on Raman peak intensity values and band areas. Pudlas *et al.*[50] conducted Raman spectroscopy to discriminate human bone marrow mesenchymal stem cells (BM-MSCs) and fibroblasts with PCA. This work can be useful in the detection of fibroblastic contamination of MSCs culturing, which contributed to the most impurity of BM-MSCs during culturing. Chen *et al.*[51] applied Raman spectroscopy to the measurement on human umbilical cord blood mesenchymal stem cells (hUC-MSCs) to determine the viability. The results showed that the lipids' structural change and destruction of DNA stacking could be reflected in Raman intensity changes. Besides, Harkness *et al.*[52] demonstrated that Raman spectroscopy could be employed to identify differences between normal and abnormal transformed MSCs. Using the PCA-LDA and leave-one-out cross validation (LOOCV) strategies, a sensitivity of 95% for classification was achieved in this study.

2.4 Corneal Infection Diagnostics

2.4.1 Corneal infection

Corneal infection refers to the infection of cornea which is a clear and transparent section in front of the eyeball. The infections are usually caused by microorganism such as virus, bacteria, fungus and parasites. Without suitable and prompt treatment tailored for particular microorganism species, corneal infection can lead to impairment in sight or even blindness.[53] Therefore, rapid and accurate diagnosis is important for corneal infection treatment.

2.4.2 Current diagnostic techniques

Currently, the standard method in clinics for corneal infection is to illuminate the suspicious region on the cornea and examine it with a special microscope named slit lamp. If the ophthalmologist suspects that the damage may be caused by an infection, a tiny tissue or scraping will be taken and then stained by pathological dyes such as Gram and Giemsa to identify the category. In case of early infection where the microorganism concentration is relatively low, microbiology culturing will be conducted to reproduce the pathogen to a detectable concentration.[54] *In vivo* confocal microscopy has been employed to improve the lateral and axial resolution to assist the diagnosis.[55] In contrast to conventional slit lamp microscopy, confocal microscopy focuses on a single spot instead of all points in the specimen. By scanning an illuminating point source, confocal microscopy can achieve optical sectioning where the depth profile of the cornea can be obtained.[56] Based on this performance, confocal microscopy achieved higher sensitivity than slit lamp. Although confocal microscopy has made progress in corneal infection diagnosis, the performance still relies on the skill and experience of the operator. Moreover, both slit lamp microscopy and current confocal microscopy [55, 57] show low accuracy in the determination of the agent of infection especially in the early stage[58] and their resolutions are still low for bacterial visualization. Thus, microbiological investigation should be always performed to confirm the species of the infectious pathogen, particularly for fungal infection[59]. Unfortunately, the incubation of fungal culture media may require up to 4-6 weeks, which is extremely time consuming. In contrast to the conventional culture

technique, PCR show significant advantages in terms of time.[60, 61] However, the results of PCR show low specificity, although the sensitivity is comparable to the culture procedure. Therefore, the relatively high possibility of false-positive results should be cautioned, though PCR appears to be promising.

2.5 Malaria Infection Diagnostics

2.5.1 Status of Global Malaria Infection and its Life Cycle

Malaria has long persisted as a leading global health problem primarily prevalent in tropical and subtropical regions. As of 2015, 212 million cases and 429,000 deaths were reported worldwide.[62] Malaria infection causes symptoms including fever, sweating, chills and headache. It takes an incubation period about 7 to 30 days for the first symptom to appear after the bite by mosquito hosting malaria parasites. However, it can exacerbate into fatal infection within hours after the first symptom.[63] Among all malaria species, malaria infection with shorter incubation period is associated mostly with *P. falciparum*. During the life cycle in human blood, malaria parasites in parasite-infected erythrocytes generate a unique pigment, *i.e.* hemozoin, in food vacuole through digestion of hemoglobin as a disposal byproduct. This malaria pigment has been frequently used as a biomarker in malaria detection.[64] Malaria parasites grow through several life stages in human blood stream, including the merozoite stage, ring stage, trophozoite stage and schizont stage. The entire life cycle takes around 48 hours. Normally, the ring stage is treated as the early stage, in which the parasites are young and the DNA shows up like a ring after Giemsa staining.

2.5.2 State of Art Techniques for Malaria Diagnosis

Although widespread and potentially fatal, prominent reduction in communal malaria transmission and full recuperation afterwards is almost guaranteed by early diagnosis and treatment. However, the gold standard of detection, namely light microscopy of Giemsa stained blood smears, remains the conventional choice despite its limitations. Due to this method's subjective nature, the accuracy of this method heavily depends on the operators' skill level especially when diagnosing infection in the early stages.[65] A seasoned technician can reportedly detect down to 5 parasites/ μL while one of average experience detects only 50-100 parasites/ μL . [66] Moreover, microscopy is inherently laborious and impractical for high-throughput examination required for mass sample sets. These problems are further compounded in remote regions where the necessary expertise or microscope is unavailable.[67, 68]

Numerous approaches have been explored to develop diagnostic tools that are more reliable and/or sensitive than the current gold standard. These include rapid diagnostic tools (RDTs)[69], cell dyn machine[70], magneto optical technology (MOT) using polarized light[71], laser desorption mass spectrometry (LDMS)[72], magnetic resonance[73], polymerase chain reaction (PCR)[74, 75], quantitative buffy coat method[76], flow cytometry[77], serological tests[78], optical tweezer[79] and attenuated total reflectance infrared spectroscopy (ATR-IR)[80], *etc.* A number of these methods are suitable to be applied in low-resource clinical settings but often do not have the desired sensitivity level needed. These include RDT, CDM, QBC, MOT and a few methods based on single cell measurements as described below. All the approaches

show reduction in sensitivity or specificity or both particularly at low parasitemia levels. The sensitivity and specificity of RDT can be over 90%, but the accuracy will decrease significantly when the parasite density is lower than 500 parasites/ μ L[69]. In addition, it is difficult to use RDT to quantify the parasitemia level.[69] The sensitivity values of cell dyn machine (CDM) and quantitative buffy coat (QBC) methods are only 50% (for parasitemia levels lower than 0.1%) and 33.3% (for parasitemia levels around 0.02%), respectively. MOT using polarized light requires a strong magnetic field while the sensitivity and specificity are only 26.7% and 80.6%, respectively, in a field test on 46 patient showing uncomplicated malaria infection detected by clinical diagnosis[81]. Flow cytometry can only measure a parasitemia level of 0.1% or higher in culture.[77]

Alternative approaches that can achieve high sensitivity at low parasitemia levels are predominately not suitable for field use due to high cost or low throughput. Laser desorption mass spectrometry is highly sensitive but is expensive and requires extensive sample preparation[72]. Serological tests can achieve high sensitivity and specificity (91% and 86%, respectively) but are time consuming and require expertise in sample interpretation [78]. Similarly approaches using optical tweezers are very time consuming and are therefore not suitable for a large volume of samples[79]. Confocal Raman spectroscopy has been conducted to observe hemozoin crystals in the vacuoles of parasites but again its throughput is low[82]. Conventional MRI offers a non-invasive method to illustrate the pathological features of cerebral malaria[83] but its operation cost is high. To overcome the high cost and bulky equipment limitations of the conventional MRI, a micromagnetic resonance relaxometry (MRR) method using a

simple low cost and portable MRI device has been reported[84]. MRR can achieve fast and quantitative measurements of the parasitemia level as low as 0.0002% for *P. falciparum* infection but the interference of hemoglobin may affect the accuracy of MRR[84]. PCR is sufficiently sensitive to detect cells infected by a single parasite and track the stage change during parasite development in real time[74]. Importantly it can differentiate between different species of human malaria parasites[85]. However, PCR still has to be seen as time-consuming and sample preparation is still relatively complicated even with the recently developed LAMP based isothermal PCR[86]. ATR-IR has even achieved a sensitivity comparable to PCR[80]. This technique can detect blood infected by parasites in the ring stage with a parasitemia level at 0.00001%_and it has been also used to quantify the parasitemia level. However, the actual sensitivity in quantification is only 0.1% and the standard deviation of the estimated parasitemia level is as high as 0.05%. Moreover, it requires an expensive diamond crystal accessory. The lowest detection limit of SERS in the current literature is about 0.0005%, which was achieved using a gold coated substrate based on butterfly wings[87]. However, this method requires the development of a cost-effective and reproducible method to fabricate complex butterfly wing nanostructures. Moreover, Raman measurements have to be taken from specific locations where the aggregation of hemozoin yields maximum enhancement. This makes it time consuming at a low parasitemia level, since hemozoin spreads sparsely on the substrate when the parasitemia level is low. All the methods applied in malaria diagnosis and their sensitivities are illustrated in Figure 1 for direct comparison.

Figure 2.1 Comparison of the current techniques for malaria diagnosis in terms of sensitivity.

Chapter 3: Raman Spectroscopy for Differentiation between Neural Progenitor Cell and its Lineages

3.1 Raman Spectroscopy in Neural Stem Cell Detection

In addition to ESC and MSC studies introduced in section 2.3, Raman spectroscopy has been conducted to detect neural stem cells (NSC). Ghita *et al.*[88] compared the Raman spectra of undifferentiated mouse neural stem cells with glial cells. Using the leave-one-out cross validation method jointly with LDA, the discrimination between neural stem cells and glial cells was achieved with 89.4% sensitivity and 96.4% specificity. They also found that the cytoplasmic RNA contributed to the major difference in spectra. To enhance the signal of cells, El-Said *et al.*[89] applied surface-enhance Raman spectroscopy on mouse neural stem cell under differentiation for over 28 days, in which a gold nanostar array was used for surface enhancement. The results showed that the percentage of DNA/RNA and total protein component increased with the differentiation time.

Although some progress has been made in neural stem cell detection, there are still two gaps to fill. Firstly, spontaneous Raman spectroscopy has not been studied systematically for the discrimination of neural progenitor cells from all lineages. Second, the system parameters during spectral acquisition vary in different studies, even Raman spectroscopy has been utilized to identify stem cells and monitor the differentiation. There is lack of optimization in system parameters to achieve the best performance to guide the use of Raman spectroscopy in this application.

Here we report the differentiation of neural progenitor cells and their lineages including oligodendrocyte progenitor cells (OPC), astrocytes and oligodendrocytes (OL) using spontaneous Raman spectroscopy. Furthermore, we evaluate the influence of system parameters during spectral acquisition on the quality of measured Raman spectra and the accuracy of classification using the spectra, which yield a set of optimal system parameters facilitating future studies.

3.2 Methodology

3.2.1 *Cell culture and sample preparation*

Rattus neural stem cells (NSC) was purchased from Gibco (N7744200) and cultured according to the manufacturer's instruction. Briefly, tissue culture polystyrene (TCPS) was coated with 20 µg/ml of Poly-L-ornithine (PLO, Sigma, St. Louis, USA), incubated overnight at room temperature and rinsed with D-PBS before use. NSCs were plated at a density of 0.5×10^5 cells per cm² in DMEM/F12 supplemented with GlutaMAX, SFM supplement, basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF) (all from Gibco, Langley, Oklahoma, USA). Upon confluency, cells were detached off the plate with pre-warmed accutase and then centrifuged into cell pellet and resuspended in a small volume of DMEM (Gibco, Langley, Oklahoma, USA) without glutamine and sodium pyruvate (DMEM w/o G&SP) to ensure high cellular concentration for experiment.

As for rattus OPC, OL and astrocyte culture, the isolation of primary culture was approved by the Institutional Animal Care and Use Committee (IACUC) at Nanyang

Technological University, Singapore. Mixed glia culture was isolated from P0-P2 neonatal rats according to a reported protocol[90]. Briefly, the cortices of rats were digested with Trypsin/DNase mixture at 37 °C for 15 mins. Cell suspension was passed through 70-µm cell strainer and the resulting solution was seeded in T75 flasks (Sigma, St. Louis, USA) in DMEM20S (DMEM w/o G&SP, 20% FBS, L-glutamine, sodium pyruvate, penicillin and streptomycin (P&S)). Fresh medium was replaced every 2-3 days. After 9-10 days in culture, OPCs were obtained by 1 hour of shaking on an orbital shaker to remove contaminating microglia followed by 18-20 hours of shaking at 37 °C. OPC was further enriched by a differential adhesion method on an untreated petri dish for an hour to remove contaminating microglia and astrocytes before filtering through 20-µm nylon screening mesh (Sefar, New York, USA) and then centrifuged into cell pellet before resuspending in small volume of PBS for experiment. The purity of OPC was more than 94% according to NG2 immunohistochemistry.

Oligodendrocyte (OL) was obtained through the differentiation of OPC. Purified OPC was seeded on Poly-D,L-Ornithine (PDLO) (Sigma, St. Louis, USA) coated glass coverslips and cultured in 50:50 DMEM high glucose: Neurobasal, B27 (Gibco, Langley, Oklahoma, USA), N-acetyl cysteine, D-biotin, ITS liquid supplement, BSA fraction V, Progesterone, Putrescine, Tri-iodothyroxine (T3) and L-Thyroxine (T4) (all from Sigma, St. Louis, USA). In the absence of mitogenic growth factors and in the presence of T3 and T4, OPC readily differentiated into MBP+ OL after 2-3 days. At least 70% MBP+ cells were observed with immunohistochemistry after 7 days in culture. OL was detached off the glass coverslip with 0.25% Trypsin and centrifuge into

cell pellet before resuspending in a small volume of PBS for experiment.

Astrocyte culture was obtained after removal of OPCs from mixed glia culture. After 18-20 hours of orbital shaking, culture flask was further shaken to remove any leftover OPC in the culture. The remaining astrocyte layer was cultured in DMEM with 10% FBS and 1% P&S. The astrocyte was at least 90% pure, confirmed by GFAP immunohistochemistry. On the day of experiment, astrocyte was detached with 0.25% Trypsin and centrifuged into cell pellet before resuspending in a small volume of PBS for experimental use.

3.2.2 Raman measurements and data processing

Raman spectra were acquired by a micro-Raman spectrometer system (inVia, Renishaw, Aberdeen, UK) coupled with a microscope (Alpha 300, WITec, Ulm, Germany) in a backscattering geometry. The system was coupled to a Czerny-Turner type spectrograph ($f = 250$ mm) with a holographic grating (1800 grooves/mm) and a RenCam CCD detector (inVia, Renishaw, Aberdeen, UK), which yields a spectral resolution of 2 cm^{-1} . A 785-nm diode laser (Renishaw, Aberdeen, UK) was used for excitation with a focal spot size about $2\text{ }\mu\text{m}$ on the sample through a microscope objective lens (50X, NA=0.65, Leica, Wetzlar, Germany). Cell suspension was smeared on an aluminum covered glass slide and then measured. In the first strategy, 10-second exposure time and 50-mW excitation power was used to measure the spectra of all types of cells including NPC, OPC, oligodendrocytes and astrocytes. Then in the second strategy, astrocytes were measured with an excitation power varying from 1 mW to 50 mW and an exposure time changing from 1 second to 10 seconds, in order to identify

optimal parameters.

For the measurements of every cell, we initially searched in the bright-field imaging mode to locate the cell body and then three spectra were obtained from the center, peripheral region and the membrane, respectively. Then these three spectra were averaged to represent one cell. In the first strategy, multiple cells were measured for each type. The background spectrum was acquired from PBS on aluminum substrate and then subtracted from all cell spectra. Subsequent data processing was performed using MATLAB. In each raw spectrum, a fifth-order polynomial was used to fit the fluorescence background and then the polynomial was subtracted from the raw spectrum to generate the clean Raman spectrum. Then the standard deviations at all wavenumbers were used to create the shadow area in Fig. 3.1, in which the height of the shadow above or below the spectrum is exactly one standard deviation at every corresponding wavenumber. To classify the spectra, PCA-LDA was performed on measured Raman spectra, using MATLAB. To be specific, PCA was first conducted to reduce the dimensions of the data. And then paired *t*-tests were conducted between every two different cell types to evaluate the differences in the first 10 principal component (PC) scores. Subsequently, PC1, PC2, PC3, PC4, PC5 and PC8 that show *p* values smaller than 0.05 in at least three pairs of *t*-tests were selected and used for LDA. For each spectrum, the results from LDA showed the possibility of classification for each cell type and then the spectra were classified as the type with the highest possibility.

3.3 Results

Figure 3.1 Raman spectra of NPC, astrocyte, OL and OPC, respectively, from the bottom curve to the top curve. The shaded region represents the respective standard deviation (Mean $\pm$ 1SD).

Figure 3.1 demonstrates the average Raman spectra of NPC, astrocyte, OL and OPC. The numbers of cells measured for NPC, astrocyte, OL and OPC are 30, 27, 31 and 36 respectively. Comparing the spectral features among all cell types, it can be observed that most bands appear in all cells, including 1660 cm^{-1} , 1452 cm^{-1} , 1341 cm^{-1} , 1268 cm^{-1} , 1127 cm^{-1} , 1004 cm^{-1} , 855 cm^{-1} , 522 cm^{-1} , 456 cm^{-1} and 426 cm^{-1} . Some spectral characteristics dominated by protein features such as the amide I band (1660 cm^{-1}) and molecular vibration such as CH₂ Wag band (1452 cm^{-1}) look very similar. Besides, the spectral region from 600 to 800 cm^{-1} representing DNA/RNA shows a bit stronger intensity in NPC spectra compared to the spectra of others types of cells. The

difference should also be noted that the peaks indicative of lipids at 786 and 1575 cm^{-1} and phospholipids at 1065 and 1127 cm^{-1} are more significant in NPC spectra than the spectra of its lineages.

Table 3.1 Classification of all cell spectra acquired from NPC, astrocyte, OL and OPC.

The reference cell type is also included to indicate the accuracy of classification.

Reference Classification	NPC	Astrocyte	OL	OPC
NPC	30	0	0	0
Astrocyte	0	23	0	0
OL	0	0	29	4
OPC	0	4	2	32

Figure 3.2 Plot of the second PC (PC2) and the fourth PC (PC4) from all cell spectra.

After data processing with the PCA-LDA method, all cell spectra can be classified to one cell type. Table 3.1 shows the results of classification for all spectra. Comparing the results with the reference type, the accuracy of classification can be easily calculated, which is 100% for NPC, 85.2% for astrocyte, 93.5% for OL and 88.9% for OPC. Moreover, the second PC (PC2) (contributing to 14.2% of the total variance) and fourth PC (PC4) (contributing to 1.9% of the total variance) that show the lowest p values in the *t*-tests are plotted in Fig. 3.2, in which we can clearly differentiate NPC from its lineages, while OL and OPC considerably overlap with each other.

Figure 3.3 Raman spectra of NPC measured with the excitation wavelengths of 514 nm (blue curve), 633 nm (green curve) and 785 nm (red curve), respectively.

Subsequently, to improve the performance of Raman spectroscopy in detection of NPC and its lineages, the optimal excitation laser and parameters including the

excitation power and exposure time were studied. In Fig. 3.3, the Raman spectra of NPC were obtained using different excitation wavelengths. An excitation power of 50 mW and an exposure time of 10 s were maintained unchanged for all measurements. Based on the characteristics of Raman spectra, the spectra acquired at an excitation wavelength of 785 nm show more prominent peaks in nearly all vibrations than those at two other excitation wavelengths, in which the peak intensity of 785-nm excitation is around 3 to 6 times those of 633 nm and 514 nm for excitation. Moreover, some peaks such as 1210 cm^{-1} only show up in the spectrum of 785-nm excitation. However, it needs to be marked that the fluorescence background in the spectrum of 785-nm excitation is higher than that of 633-nm excitation. Although we have included background removal step in data processing, some residual background may remain and affect the classification. It can be concluded that the spectrum acquired with 785 nm laser illustrated the best cell characteristics out of all the three excitation wavelengths in terms of the peak intensity and contrast.

Figure 3.4 Raman spectra of astrocytes measured using 785-nm excitation with the power varying from 1 mW to 50 mW and the exposure time from 1 second to 10 seconds.

With the confirmed optimal excitation wavelength, to further investigate the

parameter configuration of Raman spectroscopy on neural stem cells, we used the combination of varying excitation power and exposure time and compared the spectra obtained. In Fig. 3.4(a) and (b), we cannot obtain meaningful cell spectra with relatively low power and short exposure time, *e.g.* the spectrum acquired with the configuration of 1 mW and 1 second shows nearly no cell vibration bands while the spectrum with the configuration of 5 mW and 5 seconds loses some bands in the region from 800-900 cm^{-1} and 1000-1100 cm^{-1} . The spectrum with the highest maximum intensity was taken with 50-mW excitation power and 10-second exposure time, which is also the configuration used in the previous step. In Fig. 3.4, we displayed the raw intensity without calibration to facilitate the comparison of signal strength in terms of counts detected by the CCD detector since all measurements are eventually quantified in terms of counts and the maximum number of counts is fixed regardless of the excitation wavelength. It needs to be mentioned that we did not use a power higher than 50 mW and an exposure time longer than 10 seconds, in order to avoid thermal accumulation in cells[91], which will affect cell viability.

3.4 Discussion

The aim of this study is to investigate the application of Raman spectroscopy in discrimination between neural stem cells and their lineages and further optimize the configuration for the detection of neural stem cells using Raman spectroscopy. The spectra we acquired are comparable with those in the literature [88]. It can be seen from the spectra that the vibration bands of lipids (786, 1065, 1127, 1575 cm^{-1}) are

significantly weaker in the lineages, while the peaks attributed to DNA/RNA (from 600 to 800 cm^{-1}) are only slightly weaker in the lineages. The results indicate the potential of taking lipids and DNA/RNA as the biomarker during the development of neural stem cells. Besides the difference in spectral characteristics, PCA can be utilized to reduce the dimension of data and then six PCs will be processed by LDA. With the PCA-LDA method, cell spectra can be classified with high accuracy. The accuracy for NPC reaches 96.7% while those for other types of cells are all higher than 70%. Actually, the relatively low accuracy for OL can be explained by the similarity in the biological status between OPC and OL. Although we tried to purify the cells we measured, the OL pellet can still contain part of OPC, since they were differentiated from OPC. Actually, how to fully control the differentiation of OPC is still a puzzle in the field[92].

To optimize the configuration of Raman measurements on neural stem cells, we tried different excitation wavelengths and a range of values for the excitation power and exposure time. The comparison among all the spectra suggests that 785 nm is the best excitation wavelength; moreover, the configuration of 50-mW excitation power with 10-second exposure time show the most distinct characteristics. It needs to be noted that many factors can alter experimental conditions in our experiments. Most importantly, the spectra were likely not obtained from the same cell at different excitation wavelengths, although they were from the same type. It's known that the variation from different cells in Raman intensity is large. Moreover, the focal spot size of the laser varies with wavelength, which can cause difference in power density. Additionally, the Raman signal of a biological sample is always superimposed on the

fluorescence background from the same sample. The shorter excitation wavelength generates higher fluorescence that can immerse a much weaker Raman signal even the Raman signal is already stronger than that at a longer excitation wavelength. This makes it more challenging to identify Raman peaks for a shorter excitation wavelength. Therefore a longer excitation wavelength such as 785 nm is in general preferred in cell measurements. Besides, it is reasonable to believe that a power greater than 50 mW and an exposure time longer than 10 seconds could generate better Raman spectra. However, to avoid the potential thermal damage to the cells[91], we did not try higher power or longer exposure time.

The results described in the study suggest that Raman spectroscopy can be useful in the discrimination of neural stem cells from its lineages. This study can be further extended to investigate Raman spectroscopy in a more realistic setting in order to facilitate neural stem cell research. For example, all cells were deposited on an aluminum substrate in this study. In a cell therapy study, people may need to culture and differentiate cells on a fibrous scaffold [93]. In this situation, in order to obtain cell spectra on scaffold, the spectra of scaffold with biochemical coated on the surface need to be measured beforehand and subtracted from the cell spectra to get the clean cell spectra.

Despite the progress we have achieved in this study, there are still some limits to be overcome in the future. One potential improvement is to incorporate a robust bright imaging or scanning mode during Raman measurements to help identify cell locations. The focal spot in the system we used is only about 2 μm , so there is a high chance to

miss cells, especially in samples with a low cell density. Another practical problem is to measure cell spectra without detaching cells from the scaffold, in which case the signal background from the scaffold may influence the visibility of cell spectra. A special method will be required to remove such a signal background from the scaffold.

3.5 Conclusion

In conclusion, the results of our study prove the capability of Raman spectroscopy in the discrimination of NSCs from their lineages. With the PCA-LDA method, the classification of cell spectra can be very accurate. Moreover, we showed the comparison among spectra acquired with different configurations in Raman measurements and provided guidance for parameter selection related to laser wavelength, excitation power and exposure time in the configuration of Raman measurements.

Chapter 4: Raman Spectroscopy for Corneal Infection Diagnosis

4.1 Introduction

Based on the background about corneal infection introduced in Chapter 2, a non-invasive and fast diagnostic technique is important for corneal infection diagnosis. As a promising technique, Raman spectroscopy has been proved useful in the characterization of biological samples such as corneal tissues and various microorganisms including fungi, as surveyed below.

4.1.1 *Raman spectroscopy for fungi detection*

Raman spectroscopy has been used to detect and classify fungi species in the literature. Edwards *et al.*[94] reported Fourier transform Raman spectra of cell wall of three species of fungi including *Agaricus bisporus*, *Mortierella* genus and *Mucor* genus for the first time. Their preliminary results indicated the possibility of using Raman spectroscopy for discrimination between different fungi species. Maquelin *et al.*[95] used Raman spectroscopy in a prospective clinical study to identify bacterial and fungal pathogens in bloodstream infection. They created a reference library of spectra first from pathogens prevalent in bloodstream infection and then developed identification models based on linear discriminant analysis and artificial neural networks. High identification accuracy of 92.2% was demonstrated in the results. To investigate the biological composition of fungi, Gussem *et al.*[96] applied Raman spectroscopy to the analysis of four species of *Lactarius* spores—*Lactarius controversus*, *Lactarius lacunarum*, *Lactarius quieticolor* and *Lactarius quietus*. They compared the spectra of

spores with those of reference substances known to exist in macrofungi and found out that lipids contributed significantly to fungi spectra. As for the field usage, Ghosal *et al.*[97] reported the application of Raman microspectroscopy to incorporate a reference library of Raman spectra from a few types of fungi typically associated with indoor contamination and moisture-related building damages. The reference library was then used for comparison with spectra measured from individual spores and species identification. This study also evaluated different substrate and demonstrated the detection of microspores around 2 μm in air borne samples.

4.1.2 Raman spectroscopy for cornea measurement

Raman spectroscopy has been demonstrated in cornea measurements for tissue characterization. Decades ago, Goheen *et al.*[98] utilized Raman spectroscopy to investigate corneal collagen when temperature changes. Based on the results from comparison among spectra from cornea treated with temperature varying from 50 degrees to 70 degrees and aging up to 30 hours, they suggested that the swelling of cornea induced by heating and aging was primarily caused by water absorption and storage between collagen fibers. Related to this study, Fisher *et al.*[99] used Raman spectroscopy to quantify corneal hydration and assess transient changes. The results showed linear correlation between OH/CH Raman band intensities and the percentage of acetone used to treat bovine cornea. Moreover, Bauer *et al.*[100] developed *in vivo* confocal Raman spectroscopy to investigate cornea hydration. High signal-to-noise ratio spectra were achieved using an excitation laser with a wavelength of 632.8 nm and a power of 15 mJ combined with an objective lens (25X magnification, NA = 0.5,

f = 10 mm). By using the ratio of intensity between OH band and CH band as a factor, qualitative change in hydration can be observed as a result of applying dehydrating agent.

Although Raman spectroscopy has been shown effective in the detection of fungi and corneas separately, no systemic study on corneal infection by fungi has been demonstrated. Here we report the classification of corneal infection caused by three species of fungi, *i.e.* *Aspergillus fumigatus*, *Fusarium solani* and *Candida albicans*, which are the prevalent fungal pathogens in corneal infection. We first measure the Raman spectra of fungal spores from all these species using a confocal Raman spectroscopy and then conduct Raman measurements on infected rabbit corneas. The results demonstrate high accuracy in classification among three species using Raman spectra combined with principal component analysis and linear discriminant analysis.

4.2 Methodology

4.2.1 Fungi culture

The fungi culture is conducted following the published protocol[101]. For *Aspergillus* and *Fusarium* culture, 200- μ l spore suspension was taken from the stock culture and spread onto the surface of Potato Dextrose Agar (PDA) gently with a bent inoculating loop. The agar was then sealed into plates with Parafilm, and incubated at 25°C for 3 to 7 days until a lawn of mold spores was developed on the surface of the agar. To harvest the spores, 5-ml saline solution was gently dropped onto the surface of the mold spore lawn. The spores in saline solution were collected using a plate counting

method and serially diluted in distilled water. Subsequently, 0.1-ml aliquot of the diluted suspension was placed on the surface of a new PDA. The aliquot was spread evenly over the surface of the agar using a loop or glass rod. Then the PDA was incubated in plates at 25°C for 48 hours or longer until mold colonies were visualized and counted. Typically, the suspensions of spores collected were at a concentration of 10^6 to 10^7 cfu/ml. Centrifuging was conducted to obtain a higher density.

For *Candida albicans* (C. a.) culture, the spores collected from the stock was cultured on Sabourauds Dextrose Agar instead and incubated at 37 °C for 24 hours. Then 2-3 colonies were picked with a loop and dissolved in saline for the desired density.

Yong New Zealand White Rabbits (Weight varying from 1.5 kg to 2.5 kg) purchased from the National University of Singapore were used for this study and all animal experiments were conducted in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits were locally immunosuppressed by injecting Dexamethasone (Dexamethasone, Giemsa stain, Sigma, St. Louis, USA) 0.8mg/eye/day sub conjunctiva for 5 days pre-inoculation. Rabbits were anesthetized by injecting intramuscularly the mixtures containing Ketamine (50mg/kg) (Ketamine, Sigma, St. Louis, USA) and Xylazine (5mg/kg) (Xylazine hydrochloride, Sigma, St. Louis, USA). Then 1% xylocaine (Xylocaine Ointment, Guardian, Singapore) was used to anesthetize the rabbit cornea before fungi inoculation. Pre-inoculation slit-lamp examination was done for checking the corneas free from infections and inflammation. Then 10 µl of the prepared fungal solutions ($\approx 1.2 \times 10^5$ cfu/ml) was injected into the corneal stroma by using insulin syringes (30G,

Becton Dickinson, New Jersey, USA) with the aid of dissecting microscope.

After the infection, the infectious status was confirmed by slip lamp examination and then the rabbit eyes were harvested and deposited in a container for one hour prior to Raman measurements.

4.2.2 *Raman measurements and data processing*

Raman spectra of fungi spores were acquired by a micro-Raman spectrometer system (inVia, Renishaw, Aberdeen, UK) coupled with a microscope (Alpha 300, WITec, Ulm, Germany) in a backscattering geometry. The system was coupled to a Czerny-Turner type spectrograph ($f = 250$ mm) with a holographic grating (1800 grooves/mm) and a RenCam CCD detector (inVia, Renishaw, Aberdeen, UK), which yields a spectral resolution of 2 cm^{-1} . A 785-nm diode laser (Renishaw, Aberdeen, UK) was used for excitation with a focal spot size about $2\text{ }\mu\text{m}$ on the sample through a microscope objective lens (50X, NA=0.65, Leica, Wetzlar, Germany). Fungi suspension was smeared on an aluminum covered glass slide and then measurements were performed on individual spores with an excitation power of 50 mW and an exposure time of 10 seconds. The excitation power and exposure time values were selected to enhance the spectral intensity without inducing thermal damage to fungi [102].

For the measurement of fungi suspension, the spores were maintained moist and confirmed motionless. Then one spectrum was obtained from every single spore and in total ten spores were measured. Then these ten spectra were averaged to represent one fungal species. The background spectrum was acquired from PBS on aluminum

substrate and then subtracted from all fungus spectra. Subsequent data processing was performed in MATLAB. In each raw spectrum, a fifth-order polynomial was used to fit the fluorescence background and then the polynomial was subtracted from the raw spectrum to generate the clean Raman spectrum. To classify the spectra, PCA-LDA was performed on clean Raman spectra, using MATLAB. To be specific, PCA was first conducted to reduce the data dimension. Then the first six PCs accounting for most variance and showing most p-values smaller than 0.05 in all pairs of t-tests between different fungi in the original data were selected for use in LDA. LDA yielded the probability for a given spectrum belonging to each fungal species and then the spectrum was classified as the fungal species with the highest probability. All spectra were classified in one step thus this is also called one-step classification.

The infected rabbit corneas were measured by another Raman system (InnoRam-785S, B&W Tek, Newark, USA) coupled with a microscope (BAC151A, B&W Tek, Newark, USA). A 785-nm diode laser was used for excitation with a focal spot size about 100 μm on the cornea through a microscope objective lens (20X, NA = 0.4). The large focal spot in this system inferred that the measurements on corneas were obtained from a larger area compared to those using the confocal Raman microscope. Such a larger focal spot would be more desirable in future clinical measurements for the safety reason.

A total of 20 spectra were acquired from two rabbit eyes for healthy corneas, *C. a.* infected corneas and *A. f.* infected corneas. And 23 spectra were obtained from two rabbit eyes for *F. s.* infected corneas. Subsequent data processing to remove

fluorescence background and to classify the spectra were performed using the same methods for fungi suspension as described in the previous paragraph, i.e. one-step classification. In addition to one-step classification, we also tried step-wise classification. In this scheme, the four cornea groups were first divided into group 1, which contained healthy corneas and *F. s.* infected corneas, and group 2, which contained *C. a.* infected corneas and *A. f.* infected corneas. In the first step, each spectrum was firstly assigned to group 1 or group 2 by performing PCA-LDA. Then in the second step, the spectrum was further classified to an individual category in group 1 or group 2. As a consequence, a total of three classifiers were trained and used in step-wise classification to classify four cornea groups. In contrast, in one-step classification, only one classifier was used to classify all the spectra.

4.3 Results

Figure 4.1 Slit lamp images of rabbit eyes infected by (a) *Fusarium solani* (*F. s.*), (b) *Candida albicans* (*C. a.*) and (c) *Aspergillus fumigatus* (*A. f.*). The infected regions are marked by black arrows.

Figure 4.1 shows the images taken by a slit lamp on rabbit eyes infected by *F. s.*

(left), *C. a.* (middle) and *A. f.* (right). It can be seen that freckles indicating infection appeared on the surface of all corneas and the freckle pattern on *C. a.* infected cornea is more obvious than the other two. However, it needs to be noticed that the freckle distribution is random and the physical change of an infected cornea such as transparency can vary with individual cornea and infection time. Therefore, ophthalmologists discover fungal infection primarily according to symptoms and freckles but they can hardly determine the exact pathogen only by observing slit lamp images.

Figure 4.2 Raman spectra from *Aspergillus fumigatus* (A. f.), *Fusarium solani* (F. s.) and *Candida albicans* (C. a.). Ten spectra were captured and averaged for each species. The shaded region represents the respective standard deviation (Mean $\pm$ 1•SD). Note that the spectra were not normalized.

Figure 4.2 depicts the average Raman spectra measured from the spores of *Aspergillus fumigatus* (A. f.), *Fusarium solani* (F. s.) and *Candida albicans* (C. a.). Ten

spores were measured for each species. The standard deviation at each wavenumber was also shown as the shadow region. Most vibrational features attributed to biochemical in fungi can be observed in the spectra of *A. f.*, which includes lipids (967, 1010 and 1385 cm^{-1}), deoxyribose (1425 cm^{-1}), amino acid (1495 cm^{-1}) and amide (1563 cm^{-1}). The spectra from different species can be differentiated directly from spectral shape even without any mathematical analysis. Further processing by the PCA-LDA method with the first six PCs yielded a perfect accuracy of classification, i.e. 100%. These results corroborate the past similar studies and suggest the great potential of detecting fungi infection in cornea using Raman spectroscopy.

Figure 4.3 Raman spectra from healthy rabbit corneas, *F. s.* infected corneas, *C. a.* infected corneas and *A. f.* infected corneas. The shaded region represents the respective standard deviation ($\text{Mean} \pm 1 \bullet \text{SD}$). Note that the spectra were not normalized.

Figure 4.3 shows Raman spectra acquired from healthy rabbit corneas, *F. s.* infected corneas, *C. a.* infected corneas and *A. f.* infected rabbit corneas. For infected

corneas measurements, all focal spots were taken from the freckles on cornea, which were the infected regions according to our microbiologist, while the focused regions on healthy corneas were selected randomly. It can be seen from the spectra that healthy corneas and infected corneas share most vibrational bands, including 412, 859, 942, 1004, 1248, 1344, 1450 and 1668 cm^{-1} . However, some characteristic peaks in healthy corneas are absent in infected corneas, such as 1034 cm^{-1} . Moreover, some peaks such as 1668 and 1004 cm^{-1} , which can be attributed to lipids and $\nu(\text{C}=\text{C})$, respectively, are considerably smaller in the spectra of infected corneas.

(a)

(b)

(c)

(d)

Figure 4.4 (a) Plot of the first PC (PC1) score vs. the second PC (PC2) score calculated for all corneas spectra. (b) Plot of the first PC (PC1) score vs. the fourth PC (PC4) score calculated for group 1 (healthy corneas and *F. s.* infected corneas) and group 2 (*C. a.* infected corneas and *A. f.* infected corneas). (c) Plot of the first PC (PC1) score vs. the second PC (PC2) score calculated for healthy corneas and *F. s.* infected corneas. (d) Plot of the first PC (PC1) score vs. the second PC (PC2) score calculated for *C. a.* infected corneas and *A. f.* infected corneas. The black lines in (b), (c) and (d) indicate the boundary between two groups in each figure.

Table 4.1 One-step classification of all spectra acquired from healthy corneas, *F. s.* infected corneas, *C. a.* infected corneas and *A. f.* infected corneas.

Reference \ Classification	Healthy	<i>F. s.</i> infected	<i>C. a.</i> infected	<i>A. f.</i> infected

Healthy	19	1	0	0
F. s. infected	1	22	0	0
C. a. infected	0	0	18	1
A. f. infected	0	0	2	19

Table 4.2 Step-wise classification of Raman spectra acquired from healthy corneas, F. s. infected corneas, C. a. infected corneas and A. f. infected corneas.

Reference Classification	Healthy	F. s. infected	C. a. infected	A. f. infected
Healthy	19	1	0	0
F. s. infected	1	22	0	0
C. a. infected	0	0	20	0
A. f. infected	0	0	0	20

Fig. 4.4 illustrates how multiple steps in step-wise classification could improve differentiation of one from another among all groups compared to one-step classification. To be specific, the scores of the first PC (PC1) (contributing 50.94% of the total variance) and second PC (PC2) (contributing 20.14% of the total variance)

generated in one-step classification, both of which show relatively small p-values in all paired *t*-tests, are plotted in Fig. 4.4(a). The scores of the first PC (PC1) (contributing 50.94% of the total variance) and fourth PC (PC4) (contributing 2.57% of the total variance) generated in the first step of step-wise classification, both of which show the smallest p-values in the *t*-test between group 1 and group 2, are plotted in Fig. 4.4(b). Similarly, the scores of the first PC (PC1) (contributing 45.67% of all variance) and second PC (PC2) (contributing 25.74% of all variance) generated in step-wise classification, both of which show two lowest p-values in the *t*-test between healthy corneas and *F. s.* infected corneas, are shown in Fig. 4.4(c). Meanwhile, the scores of the first PC (PC1) (contributing 54.47% of all variance) and second PC (PC2) (contributing 28.45% of all variance) generated in step-wise classification, both of which show two lowest p-values in the *t*-test between *C. a.* corneas and *A. f.* infected corneas, are shown in Fig. 4.4(d). These selected PCs demonstrate the most prominent difference between each pair of groups according to the p-values of *t*-test results. In Fig. 4.4(a), while healthy corneas can be well differentiated from infected corneas in one-step classification, the three infected cornea groups mix with each other significantly. This suggests that two PC scores are inadequate to differentiate among infected groups. Actually even six PC scores cannot classify all species accurately as shown in Table 4.1. The classification can be improved using the step-wise strategy. The difficulty of differentiating all species in one-step classification can be reduced by classifying two groups each time and repeating this for several times. In Fig. 4.4(b), group 1 (healthy corneas and *F. s.* infected corneas) and group 2 (*C. a.* infected corneas and *A. f.* infected

corneas) can be differentiated nicely with two PC scores, in spite of some overlaps. In Fig. 4.4(c), healthy corneas and *F. s.* infected corneas can be differentiated well in spite of some overlaps. Similarly, *C. a.* infected corneas and *A. f.* infected corneas can be differentiated well in Fig. 4.4(d).

It need to be noted that the scores of two PCs plotted here are just for illustration. In practice, two PC scores are far from enough to achieve acceptable classification accuracy. To achieve more precise classification, we utilized the first six PCs that show p values smaller than 0.05 in at least three pairs of t -tests in classification with the LDA method. Table 1 shows the results of one-step classification for all spectra. The accuracy of classification is 95% for healthy corneas, 95.7% for *F. s.* infected corneas, 90% for *C. a.* infected corneas and 95% for *A. f.* infected corneas. Table 4.2 shows the results of step-wise classification for all spectra. The accuracy of classification is 95% for healthy corneas, 95.7% for *F. s.* infected corneas, 100% for *C. a.* infected corneas and 100% for *A. f.* infected corneas. Therefore, the overall accuracy using step-wise classification is higher than one-step classification.

4.4 Discussion and Conclusion

The aim of this study is to investigate the feasibility of using Raman spectroscopy to diagnose corneal infection due to three prevalent fungi species and differentiate them from each other. Based on our results, Raman spectroscopy can be used to accurately differentiate the three fungi species in spores with perfect accuracy, i.e. 100%, in this

particular study. Furthermore, healthy corneas and corneas infected by the three fungi species in a rabbit cornea model can be classified with high accuracy, i.e. 95% for healthy cornea, 95.7% for *F. s.* infected corneas, 100% for both *C. a.* and *A. f.* infected corneas, using the multi-variate analysis of Raman spectra and step-wise classification. The label-free nature, quick measurements and high accuracy demonstrated by Raman spectroscopy suggest its great promise in corneal infection diagnosis.

To achieve the best performance of classification using the PCA-LDA method, we conducted classification in two different ways, i.e. one-step classification and step-wise classification. In the one-step classification, all 83 spectra were classified into all four different groups at once; in contrast, multiple steps are taken in the step-wise classification and only binary classification is conducted at each step. It can be seen that the accuracy achieved by the step-wise classification was higher than that by the one-step classification. This observation can be explained as follows. In one-step classification, only one classifier was trained for four groups of corneas so the classifier often needs to make a tradeoff to avoid bias towards any particular group. In step-wise classification, three classifiers were trained to differentiate between group 1 and group 2, between healthy corneas and *F. s.* infected cornea, and between *C. a.* infected corneas and *A. f.* infected corneas, respectively. In this case, each binary classifier can achieve maximal accuracy because there is no tradeoff necessary as in one-step classification. It should be mentioned that, due to the large laser focus of our system used in corneas measurements, there was a possibility that the spectra obtained from an infected cornea might contain contribution from the healthy part. This phenomenon was

more prominent in *F. s.* infected corneas, since the infected pattern on *F. s.* infected cornea was not as obvious as that on other infected corneas (see Fig. 1). To overcome this problem, an advanced data processing method is needed to remove the contribution from the healthy part in the Raman spectrum of an infected cornea. Such a method will improve the subsequent differentiation of corneal infection caused by different species.

The difference in spectra acquired among infections caused by different pathogens provides the insight of biomedical changes during infection. In this aspect, one interesting observation is that, the Raman spectrum of an infected cornea is not simply the linear summation of those of healthy cornea and fungi spores according to this study. During infection, the fungi can damage the structure of a cornea and generate various biochemical, including proteins, carbohydrates and uronic acid[59, 103], in the process. As shown in Fig. 3, in which the Raman spectra without normalization keep the original intensity values, the peak 1668 cm^{-1} attributed to lipids shows a lower intensity in the average spectrum of infected corneas compared to healthy ones, which indicates that the lipid content in the corneas decrease during fungal infection. This agrees with the previous reports in the literature[104].

One challenge for the clinical adoption of Raman spectroscopy is that the traditional Raman measurements require relatively high laser excitation power and long exposure time, which may cause damage to eyes in future *in vivo* measurements. This problem could limit its application in the field if unsolved. Some earlier reports have demonstrated various strategies to overcome the safety problem of applying lasers to the eyes. One early work applying the laser on eye surgery has demonstrated that the

transmission of laser through cornea can be minimized and the retina damage could be avoided using the excimer laser [105]. Moreover, the efficiency improvement of Raman system [106, 107] or the utilization of resonance Raman scattering [108] could also lower down the requirement on excitation power and time.

Chapter 5: Surfaced Enhanced Raman Spectroscopy for Early Malaria Diagnosis

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Spontaneous Raman spectroscopy used in the previous two chapters has an advantage in its label free nature. However, it will suffer from low sensitivity in the detection of a biomarker at low concentrations such as in early malaria diagnosis. In this situation, it is necessary to boost up the sensitivity of Raman spectroscopy with surface enhancement enabled by nanoparticles at the cost of being no longer label free. Given that malaria diagnosis is often performed on blood samples that have been taken out of human body, this is not a practical problem.

5.1 Raman Spectroscopy (RS) and SERS in Malaria Research and Diagnosis

The literature in RS and SERS for malaria research on diagnosis can be separated into two groups that focus on qualitative and quantitative studies, respectively.

5.1.1 *Qualitative Raman Measurements*

Inhibition of hemozoin formation is a prominent curative target for anti-malarial drugs such as chloroquine and quinine. As such, characterization of hemozoin crystallization using RS provides invaluable insight into drug effectiveness and feedback on drug improvement. Bonifacia *et al.*[109] visualized the distribution of

hemoglobin and hemozoin simultaneously using RRS maps of parasitized RBCs processed by hierarchical cluster analysis, which provided information about the hemozoin formation process. Wood *et al.*[110] measured the Raman spectra of β -hematin, a synthetic analogue of hemozoin possessing an identical Raman spectrum [110], and hematin with a wide range of excitation wavelengths, and found dramatic enhancement in β -hematin in comparison to monomeric hemin when using 780 nm and 830 nm. This difference in intensity is strongly attributed to the covalent stacking of hemes. Frosch *et al.*[111] selectively enhanced vibrational modes by varying excitation wavelengths to derive morphological information of hemozoin. Morphology-sensitive low-wavenumber modes residing below 400 cm^{-1} were enhanced using excitation wavelengths at 633 nm and 647 nm.

Webster *et al.*[112] exploited resonance Raman spectroscopy to evaluate the effect of chloroquine treatment on cultures of *Plasmodium falciparum* trophozoites. Using a 782-nm laser for excitation, a reduction in the intensity of Raman bands associated with hemozoin aggregates in treated cells was observed, which provided proof of this drug's negative influence on hemozoin formation. In a similar study, Kozincki *et al.*[113] used Raman spectroscopy to compare chloroquine treated and untreated cultured RBCs infected with *Plasmodium falciparum*, and revealed consistent spectral differences attributed to higher level of oxygenated hemoglobin in treated cells. These findings were confirmed by Dasgupta *et al.*[114] who investigated RBCs with Raman optical tweezers. Significant spectral differences in about 30% of RBCs from malaria infected patients indicated reduced oxygen affinity by hemoglobin. Further studies focused on

RBCs using dark-field microscopy and optical trapping. Wood *et al.*[115] successfully employed partial dark-field microscopy to locate hemozoin deposits as targets for Raman detection. Unlike bright-field microscopy where parasites are difficult to see, hemozoin deposits appeared brightly in partial dark-field microscopy. This team produced spectra with a high signal-to-noise ratio in just one second that confirmed the presence of the characteristic hemozoin peak at 1569 cm^{-1} . Reconstructed Raman images of RBCs revealed possible sub-micron-inclusions of hemozoin that may be promising for identifying miniscule amounts of malaria pigment typical in the early stages of infection. Although these inclusions could not be confirmed as hemozoin by alternate methods, the similarity in peak intensities at 1569 cm^{-1} and 1639 cm^{-1} , associated with oxygenated hemoglobin, is strong evidence supporting that conclusion.

5.1.2 Quantitative Raman and SERS Measurements

The magnetic property of hemozoin has been used to separate infected and normal RBCs.[28, 116] This property was recently utilized by Yuen et al.[28] to develop magnetic field enriched SERS, in which iron oxide core with silver shells ($\text{Fe}_3\text{O}_4@\text{Ag}$) instead of silver nanoparticles were used to further enhance SERS from β -hematin crystals through magnetic attraction. Compared to resonance Raman measurements, SERS signals taken under an external magnetic field showed about five orders of magnitude enhancement to achieve a detection limit of 5-nM of β -hematin, which is equivalent to about 30 parasites/ μL . Their further work demonstrated the ability to tune the performance of this scheme by varying the core diameter to shell thickness ratio. [116] While magnetic field enriched SERS is sensitive, the requirement of an external

magnetic field is inconvenient, which could induce additional variation in SERS readings.

Strong spectral similarity between hemoglobin and hemozoin, due to the presence of heme, stands as a significant obstacle for early detection. Hobro *et al.*[117] eliminated the strong heme background from RBCs in mice infected by *Plasmodium yoelii* by measuring RS signals from plasma samples containing hemozoin crystals released from RBCs already broken by the parasites. This enabled parasite detection just one day after infection at parasitemia levels between 0.05 and 0.2% in contrast to four days at a parasitemia level of about 3% achieved by directly probing RBCs. Unfortunately, this method was not suitable for making quantitative measurements due to the use of principal component analysis and showed no linear relation between characteristic Raman peak intensity and time after infection.

5.2 Methodology

5.2.1 *Parasite culture*

Plasmodium falciparum parasite (3D7) were cultured in fresh red blood cells (RBCs) at 5% hematocrit with media constituted of bicarbonate buffered RPMI 1640 (RPMI 1640 Medium, Life Technologies, Grand Island, USA) supplemented with 5% albumax (AlbuMAX® I Lipid-Rich BSA, Life Technologies, Grand Island, USA), 200 μ M hypoxanthine and 20 μ g/ml gentamycin as described previously.[118] The cells were maintained at 37°C and a gas mixture of 5% CO₂, 1% O₂ and 94% N₂. After 5 cycles of parasite growth, the mixed culture was synchronized using 5% D-sorbitol (D-

Sorbitol, Sigma, St. Louis, USA) treatment[119] and allowed to grow one more cycle then were washed to get rid of floating hemozoin. Parasites were mounted onto glass slides and stained with Giemsa (Giemsa stain, Sigma, St. Louis, USA). The parasitemia level was estimated by manual counting under microscopic observation after Giemsa staining and confirmed by staining cells with syber-green (SYBER®Green, Life Technologies, Grand Island, USA) dye and counting with BD LSRII FACS (BD Biosciences, San Jose, USA). Appropriate dilutions were made using non-infected blood in RPMI 140 for further analysis. Optical microscopy was used to confirm the stages of parasites. It turned out that nearly 98% of parasites were in the ring stage and 2% in the early trophozoite stage in this study.

5.2.2 Sample preparation in Method 1 (synthesizing nanoparticles separately)

In Method 1, silver nanoparticles were synthesized separately and then mixed with isolated hemozoin. Silver nanoparticles were synthesized using the reduction method.[28, 116, 120] A total of 33- μ l Triton X-100 (Triton X-100 Detergent, Bio-Rad Laboratories, Hercules, USA) was mixed with 5-ml hydroxylamine hydrochloride (0.03 mM, MP Biomedicals, Santa Ana, USA) and NaOH (0.15 mM). The mixture was added drop-wise over a period of 8 min to 45 ml aqueous AgNO₃ (Silver nitrate, Merck, Kenilworth, USA) with a concentration of 1.11 mM. The resulting solution was then sonicated (Elma E30H, Elma, Wetzikon, Switzerland) for 30 min. Finally, the generated silver nanoparticles were centrifuged and resuspended in 5-ml solution. The suspension was sonicated for 5 min for later use.

Hemozoin crystals were extracted by simple cell lysis and centrifuging. A total of 10- μ l infected blood sample were dispersed in 50-ml deionized water mixed with 100- μ l Triton X-100 and then sonicated for 5 min. Released hemozoin crystals were collected by centrifuging at 5000 rpm for 5 min (Sartorius 2-16, Sigma Laborzentrifugen, Ostrode, Germany) and resuspended in 5 ml NaOH solution (0.05 mM).

Then silver nanoparticles suspension and hemozoin suspension were mixed together (1:1 v/v). The mixture was sonicated for another 2 min. During Raman measurements, the sample was smeared on a slide covered by aluminum foil as the substrate. On each slide, five random locations were selected for Raman measurements while the sample was still wet.

5.2.3 Sample preparation in Method 2 (synthesizing nanoparticles inside parasites)

In Method 2, silver nanoparticles were synthesized inside parasites directly to achieve closer contact with hemozoin than Method 1. It should be highlighted that this method keeps the local hemozoin concentration in parasites because it does not break down parasites. A total of 10- μ l lysed blood was dispersed in 50-ml deionized water and then sonicated for 5 min to achieve blood cell lysis. Different from the cell lysis procedure in Method 1, Triton X-100 was removed in the lysis step of Method 2 to prevent the lysis of parasites. The mixture was pushed through a filter with a pore size of 0.2 μ m (Supor Syringe filters, Pall Life Science, Washington, USA) in a syringe to remove hemoglobin in the lysed blood sample. Then, the residue in the filter was

flushed out and suspended in 45-ml AgNO_3 (Silver nitrate, Merck, Kenilworth, USA) solution with a concentration of 1.11 mM. Then 33- μl Triton X-100 (Triton X-100 Detergent, Bio-Rad Laboratories, Hercules, USA) was mixed with 5-ml solution of hydroxylamine hydrochloride (0.03 mM, MP Biomedicals, Santa Ana, USA) and NaOH (0.15 mM), which was then added drop-wise over a period of 8 min to aqueous AgNO_3 to reduce silver. In this step, the volume of Triton X-100 was low and it mostly bonded to silver nanoparticles to prevent them from aggregation, which limited its effect on cell lysis to a low level. The resulting solution was then sonicated (Elma E30H, Elma, Wetzikon, Switzerland) for 30 min. Finally, the sonicated solution was pushed through a 0.2- μm filter (Supor Syringe filters, 25mm, 0.2 μm , Pall Life Science, Washington, USA) in a syringe to filter away small excessive nanoparticles and chemicals and leave analyte on the filter paper. The filter paper was taken out and deposited on a slide covered by aluminum foil for Raman measurements. For each filter paper, five random locations were selected for Raman measurements.

5.2.4 Raman measurements and data processing

A micro-Raman spectrometer system (inVia, Renishaw, Aberdeen, UK) coupled with a microscope (Alpha 300, WITec, Ulm, Germany) in a backscattering geometry was used to measure all Raman spectra. The system was coupled to a Czerny-Turner type spectrograph ($f = 250$ mm) with a holographic grating (1800 grooves/mm) and a RenCam CCD detector (inVia, Renishaw, Aberdeen, UK), which yields a spectral resolution of 2 cm^{-1} . A 633-nm diode laser (Renishaw, Aberdeen, UK) was used for excitation with a focal spot size about 3 μm on the sample through a microscope

objective lens (5X, NA=0.2, Leica, Wetzlar, Germany). The exposure time was 10 seconds and the excitation was 2.5 mW for all SERS measurements. The combination of similar exposure time and excitation power was also used previously in our magnetic enriched SERS study[28], which can achieve the tradeoff between the maximization of SERS intensity and minimization of the thermal effect.

In each sample, the Raman spectra were measured from five random locations then averaged to reduce variation. No hot spot search was done during spectral measurements in Method 1 or Method 2. The measurement at each parasitemia level was repeated in seven different samples in Method 1 and five different samples in Method 2 for every concentration to calculate the standard deviation. In each raw spectrum, a fifth-order polynomial was used to fit the fluorescence background and then the polynomial was subtracted from the raw spectrum to generate the clean Raman spectrum.

To obtain the contribution of hemozoin alone in the measurement of an infected blood sample for convenient visualization in Method 1, the clean Raman spectrum of an infected blood sample was modeled as the summation of the contribution from the normal blood sample and that from hemozoin. The Raman spectra of the latter two basic components were measured separately, in which the basic component spectrum of hemozoin was replaced by that measured from β -hematin at a concentration 10 $\mu\text{g/ml}$. This is justified by the fact that β -hematin is equivalent to hemozoin in terms of Raman features[121, 122] and much easier to synthesize. Then the contribution of each component to the infected blood spectrum was estimated using a least square regression

method[123] implemented by the lsqcurvefit function in MATLAB with an option of trust-region-reflective algorithm.

5.2.5 *Detection limit estimation*

To determine the detection limit for Method 1, a *t*-test was conducted to evaluate the difference in hemozoin contribution between every infected blood sample with a progressively decreasing parasitemia level and the normal blood sample. When there was no difference between the two samples, the parasitemia level in the previous infected blood sample that still demonstrated a significant difference in hemozoin contribution compared to the normal blood sample was determined to be the smallest detectable parasitemia level. The lowest detectable parasitemia level for Method 2 was determined in the same manner by conducting a series of *t*-tests except that the Raman peak intensity at 1623 cm^{-1} instead of hemozoin contribution was compared.

5.2.6 *Ethics statement*

The use of human blood strictly followed protocols and guidelines that were approved by the domain-specific review board of Nanyang Technological University (IRB number: NTU-IRB 11/12/2011). Blood component collection service was provided by Blood Transfusion Service and Blood Donation Centre of National University Hospital. All individuals gave informed consent.

5.3 Results

Figure 5.1 SERS spectra and hemozoin contribution in Method 1. (a) SERS spectra contributed by hemozoin in infected blood treated by Method 1. (b) Hemozoin contribution as a function of parasitemia level. In (a), the data point corresponding to a parasitemia level of 0.01% is marked by a red box. In (b), the resulting curve of the second-order polynomial fitting for the data points corresponding to parasitemia levels in the range of 0.01% to 0.2% is shown in red. The data corresponding to normal blood samples, labeled as “NB”, is added manually to facilitate comparison. The y axes in both figures are plotted in arbitrary units, labeled as “A. U.”.

Figure 5.1 shows (a) the SERS spectra and (b) hemozoin contribution as a function of parasitemia level for infected and normal blood samples obtained using Method 1. Prominent vibrational features of hemozoin, such as ν_{15} at 754 cm⁻¹, ν_2 at 1570 cm⁻¹,

and $\nu_{(C=C)}$ at 1623 cm^{-1} are shown in most of the spectra in Fig. 5.1(a). Detailed peak assignments are shown in Table 5.2. Hemozoin contribution refers to the weight of the basic component spectrum corresponding to hemozoin resulting from the least square regression as described in data processing section. The lowest detectable parasitemia level was determined to be 0.01% after conducting a series of *t*-tests to compare the hemozoin contribution in infected blood samples with that in the normal blood sample ($p < 0.05$). There appears to be a strong correlation between hemozoin contribution and the parasitemia level in Fig. 5.1(b).

Figure 5.2 SERS spectra and hemozoin Raman peak distribution in Method 2. (a) SERS spectra of normal blood and infected blood sample treated by Method 2. (b) SERS peak intensity at 1623 cm^{-1} as a function of parasitemia level. The data corresponding to

normal blood samples, labeled as “NB”, is added manually to facilitate comparison. The y axes in both figures are plotted in arbitrary units, labeled as “A. U.”.

Figure 5.2 shows the SERS spectra and ν_{10} peak intensity as a function of parasitemia level obtained using Method 2, in which silver nanoparticles were synthesized inside parasites. Compared with the spectra in Fig. 5.1, the Raman spectra in Fig. 5.2 are much stronger. Prominent vibrational features of hemozoin, such as ν_{15} at 754 cm^{-1} , ν_2 at 1570 cm^{-1} , and $\nu_{(\text{C}=\text{C})}$ at 1623 cm^{-1} are shown in all spectra. Detailed peak assignments are shown in Table 5.2. Multiple hemozoin Raman peaks are visible in the spectra of normal blood samples, which can be attributed to hematin converted from hemoglobin during sample preparation. In Method 2, most hemoglobin were filtered out, whereas a tiny amount could still stay in or attached to parasites. During silver nanoparticles synthesis, the use of detergent Triton X-100 and hydroxylamine hydrochloride likely converted the residue hemoglobin to heme and then hematin[124, 125] which contributed to the Raman peaks[122]. Note that hematin and hemozoin share most peaks including 1623 cm^{-1} . The detection limit for Method 2 is determined to be 0.00005% parasitemia level in the ring stage, after comparing the Raman peak intensity at 1623 cm^{-1} measured from infected blood samples with that from the normal blood sample with *t*-test ($p < 0.001$). The detection limit is exceptionally low compared to most relevant publications we can find (shown in Table 5.1).

Figure 5.3 Giemsa stained images of blood samples treated by Method 1 and Method 2. (a) a blood sample with parasites in the ring stage prior to cell lysis, (b) silver nanoparticles alone, (c) a blood sample after blood cell membrane lysis but prior to nanoparticle synthesis treated by Method 2, (d) the blood sample after nanoparticle synthesis treated by Method 2 and (e) a blood sample treated by Method 1 and then

mixed with nanoparticles synthesized separately.

To illustrate the difference in the separation of parasites and nanoparticles between Methods 1 and 2, Fig. 5.3 shows the Giemsa stained images of a typical infected blood sample, silver nanoparticles and a blood sample treated by Method 2 before and after nanoparticle synthesis as well as a blood sample treated by Method 1 for comparison. Figure 5.3(a) illustrates the purple rings superimposed on top of red blood cells, which are the stained DNAs of malaria parasites in the ring stage. Figure 5.3(b) displays the Giemsa stained image of silver nanoparticles serving as a reference to facilitate search for nanoparticles in subsequent images, in which aggregated nanoparticles show up as sparsely distributed brown or black dots. For comparison, the FESEM image of dispersed silver nanoparticles is displayed in Fig. 5.4 (see below). In Fig. 5.3(c), red blood cells have been lysed as treated by Method 2 so the blood cells do not exist in background, which is visibly different from Fig. 5.3(a). However, parasites were not lysed because the concentration of the lysing agent was purposefully reduced. For this reason, the stained DNA rings are clearly seen. In Fig 5.3(d), nanoparticles have been synthesized in the lysed blood sample as treated by Method 2. It can be clearly seen that some nanoparticles are in the close proximity to the DNA rings of parasites as indicated by red arrows. This phenomenon can be observed in roughly one out of every ten rings in samples treated by Method 2. This suggests that silver nanoparticles were more likely to be close to hemozoin inside the same parasites. Because hemozoin was not released to the outside of parasites, the original local hemozoin concentrations in the parasites were maintained, which created higher SERS signals than otherwise. For

the purpose of comparison, Fig. 5.3(e) illustrates the Giemsa stained image of a blood sample treated by Method 1, in which lysed blood samples and parasites were mixed with nanoparticles separately synthesized. Because parasites have been lysed, no DNA rings were observed and hemozoin inside parasites were thus released into the entire sample. Hemozoin concentration in the sample was thus much lower than that in the original parasites. The SERS signal from hemozoin was thus much lower as expected.

Figure 5.4 FESEM image of silver nanoparticles synthesized in Method 1. The nanoparticle size ranges from 50 to 100 nm.

5.4 Discussion

Table 5.1 Sensitivities of current methods in malaria diagnosis. The percent parasitemia level is converted to the number of parasites per microliter with the assumption that

normal blood in human body contains 5×10^9 RBCs/ml.

Methods	Detection limit in the ring stage	Original statement without conversion
Giemsa stained microscopy	4 to 20 parasites/ μ l in thick smear	4 to 20 parasites/ μ l in thick smear for all different stages. The threshold may be higher in the field for the early stage ^{4,5} .
Flow cytometry	100000 parasites/ μ l	This technique is useful for a parasitemia level around 2% and maybe more helpful in a late stage[77].
Magneto optical technology (MOT) using polarized light	10000 parasites/ μ l*	0.002% parasitemia level in the trophozoite stage[71].
Quantitative Buffy Coat (QBC) Test	1000 parasites/ μ l	The sensitivity will be lower than 30% when the parasitemia level is lower than 1000 parasites/ l[76].
Rapid diagnostic tools (RDTs)	100 to 500 parasites/ μ l for <i>P. falciparum</i> infection	100 to 500 parasites/ μ l for <i>P. falciparum</i> infection in all different

		stages[69, 126].
Cell dyn machine	27.786 parasites/ μ l (in unknown stages)	The parasitemia level of clinical samples varies from 320 to 285714 parasites/ml with an average of 27786 parasites/ml, using Cell dyn 3700 [70].
Laser desorption mass spectrometry (LDMS)	10 parasites/ μ l	10 parasites/ μ l in the ring stage[72].
Micromagnetic Resonance Relaxometry (MRR)	10 parasites/ μ l for <i>P. falciparum</i> infection	0.0002% parasitemia level for <i>P. falciparum</i> infection mostly in the ring stage and 0.0001% for <i>P. berghei</i> mouse model[84].
Polymerase Chain Reaction (PCR)	around 0.7 parasites/ μ l for <i>P. falciparum</i> infection	Real-time PCR assay shows a detection limit of 0.7, 4, and 1.5 parasites/ μ l for <i>P. falciparum</i> , <i>P. vivax</i> , and <i>P. ovale</i> , respectively in different stages[74, 75].

Attenuated total reflectance infrared spectroscopy (ATR-IR)	0.5 parasites/ μ l	The absolute detection limit was found to be 0.00001% parasitemia in the ring stage[80].
Surface enhanced Raman spectroscopy (SERS) using butterfly-wing nanostructures	25 parasites/ μ l	0.0005% parasitemia level in the ring stage[87].
Method 1 in our study	500 parasites/ μ l	0.01% parasitemia level in the ring stage.
Method 2 in our study	2.5 parasites/ μ l	0.00005% parasitemia level in the ring stage.

*The detection limit of the MOT method was converted from the hemozoin concentration[71] to the parasitemia level in the original paper. Their assumptions in conversion included: A) hemoglobin concentration in a healthy human is around 340 g/l; B) parasites in the trophozoite stage convert 50% of hemoglobin to hemozoin and then yield around 0.6 pg hemozoin per cell. The detection limit of 0.06 μ g/ml in hemozoin concentration was converted to a parasitemia level of 100 parasites/ μ l in the trophozoite stage.[71] For consistency with all other techniques, the parasitemia level in the trophozoite stage is converted to that in the ring stage here for the same hemozoin concentration based on the assumption that hemozoin concentration in the trophozoite stage is 100 times that in the ring stage[64, 127]. This yields a sensitivity of 10000 parasites/ μ l in the ring stage for MOT.

Table 5.2 Comparison in the peaks of SERS spectra between Method 1 and Method 2.

The peaks of spontaneous Raman spectra measured from hemozoin in parasites' vacuoles in the literature[82] and their assignment are also listed for comparison[128].

Wavenumber in Method 1 (cm^{-1})	Wavenumber in Method 2 (cm^{-1})	Wavenumber in the literature (cm^{-1})[82]	Assignment[128]
1623	1623	1627	$\nu(\text{C}=\text{C})$
1570	1569	1569	ν_2
1432	1431	1430	ν_{28}
1399	1392	1398	ν_{20}
1371	1372	1375	ν_4
1337	Absent	1339	ν_{41}
1307	1309	1308	ν_{21}
1237	1236	1239	Not available
1169	1168	1220	ν_{30}
1146	1146	1147	Not available
1124	1126	1122	ν_{22}
1091	1091	1091	ν_{23}
1053	1054	Absent	Not available

1033	1033	Absent	Not available
1003	1002	1003	V ₄₇
976	974	973	V ₄₆
938	935	942	Not available
825	823	821	Not available
793	789	797	V ₆
754	754	754	V ₁₅
714	714	Absent	Not available
676	677	Absent	V ₇

Asymptomatic patients with extremely low levels of parasitemia pose a key challenge in any effort to control malaria. The extremely low levels of circulating parasites in the peripheral blood[129] make accurate diagnosis particularly difficult. Here we present data that clearly demonstrates the feasibility and advantage of surface enhanced Raman spectroscopy for detecting malaria parasites under those circumstances. While the lowest detectable parasitemia level for Method 1 is approximately 0.01% ring stage parasites, comparable to other methods such as QBC and RDTs in malaria diagnosis (Table 5.1), Method 2 has a detection limit as low as 0.00005%, which equates to approximately 2.5 parasites/ μ l of blood and is comparable to the most sensitive detection techniques currently available. The linear correlation between hemozoin concentration and parasitemia level seen in Method 1 has the

additional advantage of allowing the direct quantification of the parasite level, which would be of great value for the rapid determination of the overall severity of malaria infection. The relatively high ratio of standard deviation to mean seen at each parasitemia level in Method 1 (15% to 58%) is likely the result of variations in contaminating cell debris that adhere to hemozoin crystals and silver nanoparticles during the centrifugation step. This contamination would directly affect the interaction between hemozoin and silver nanoparticles. In addition the lysis method using Triton X-100 and sonication may not be able to release all hemozoin out of every parasite's vacuole leading to an overall reduced signal although cost effective. Future work will focus on improving the lysis step to further increase the sensitivity of Method 1.

The exceptionally high sensitivity of Method 2 is better than most existing methods (Table 5.1). The close proximity of nanoparticles to hemozoin concentrated inside parasites achieved in this approach (Fig. 5.3) dramatically enhances the Raman signal of hemozoin leading to the improved detection limit. While very promising, one limitation of synthesizing nanoparticles inside parasites is the inconsistent distribution of accumulated hemozoin within the parasites. The Raman signal acquired from a location with parasites and nanoparticles inside would be much stronger than that from another location without them. Because the distribution of parasites with nanoparticles inside is discrete and usually inhomogeneous in the sample, the variation in Raman peak intensities from random locations or different batches is higher than that in Method 1. For the same reason, there is no strong correlation between the SERS peak intensity and the parasitemia level. However, with the lowest detectable parasitemia level at

0.00005%, Method 2 can be utilized to determine whether a patient is infection positive or to screen high-risk population.

The simplicity and speed of our SERS based techniques indicate their great promise in malaria diagnosis. In the case of *P. falciparum* infections in which parasites observed in peripheral blood are predominantly in the ring stage, Method 1 would allow the relatively accurate determination of the parasitemia level. While infections containing asynchronous stages would be more difficult to accurately quantify as the hemozoin level varies between the different stages unless an extra synchronization step is employed.

One possible challenge for SERS based techniques that rely on solely hemozoin measurements is its inability to distinguish between hemozoin produced by a viable parasite and that still circulating in blood due to a prior infection. One earlier work has shown that detection of residual hemozoin using a Cell-Dyn automated haematology analyser from a prior malaria infection can be achieved up to 4 weeks after treatment[70]. This suggests the more sensitive SERS technique would also detect hemozoin from past malaria infections, which will somewhat limit the ability of this approach to making a definitive diagnosis of an active malaria infection. However the technique can be used to accurately exclude a malaria infection and provide crucial data of either a recent or current asymptomatic infection. This information would be of immense value in the later stages of a malaria eradication campaign [130, 131].

Compared to the previously published SERS method[87], our strategy provides the

added benefit of easier nanoparticle preparation, lower cost and higher sensitivity. Moreover, the SERS measurements in our methods were performed on random locations rather than on selected hot spots resulting in increased speed. Although an automated method for hot spot searching has been developed [132, 133], such a method requires scanning the entire sample and its accuracy can be affected by fluorescence, which makes it time-consuming and inconvenient. The waiver of hot spot search in our techniques would facilitate the development of fast and convenient automated SERS measurements for field use in the future. In addition, Method 1 also provides the possibility to quantify parasitemia levels directly, which was not available in the published SERS method[87] possibly due to large variation in SERS peak intensities. While the greater variation of spectral intensities in Method 2 is one limitation, two potential strategies could be used to minimize it. One strategy is to combine Method 2 with an automated hot-spot searching method. With a proper threshold setting, one can scan the entire sample to measure only “hot spots” in the sample to minimize variation. The other potential way is to concentrate the parasites with nanoparticles using an isolating device such as MidiMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), to minimize the chance of measuring regions that contain no parasites or nanoparticles.

The improvements described here now make SERS comparable to the standard microscopy method in terms of detection limit but without the need of an experienced operator. Most steps in the two methods reported in this study could be implemented in a microfluidic chip to enable automatic sample preparation. A paper based microfluidic chip[134] could further lower the cost of sample preparation. The bottleneck in the high

cost of a Raman spectrometer with decent spectral resolution could be overcome by a much cheaper alternative method, in which a cost effective and compact Raman spectrometer[135] acquires raw Raman spectra with low spectral resolution and a spectral recovery method our group developed recently[136] is used to reconstruct the Raman spectra with high spectral resolution. Therefore the two SERS based methods developed in this study in combination with the cost effective Raman spectrometer we are currently developing hold great potential for malaria diagnosis even in low-resource regions.

5.5 Conclusion

In conclusion, the simplicity and speed of our SERS based techniques indicate their great promise in malaria diagnosis. In the case of *P. falciparum* infections in which parasites observed in peripheral blood are predominantly in the ring stage, Method 1 would allow the relatively accurate determination of the parasitemia level. While infections containing asynchronous stages would be more difficult to accurately quantify as the hemozoin level varies between the different stages unless an extra synchronization step is employed. Our methods show great potential to achieve ultrasensitive and quantitative measurement in early malaria infection.

Chapter 6: Surface Enhanced Raman Spectroscopy for Single Parasite Detection

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It has been shown in Chapter 5 that our ultra-sensitive SERS technique can achieve hemozoin detection at a very low level. The high sensitivity of this SERS technique may offer a convenient way for hemozoin detection in single parasites, which is important to research in malaria development and anti-malaria drug.

6.1 Raman Spectroscopy and SERS in Single Parasites Detection

The capability of analyzing hemozoin *in situ* within the vacuoles of parasites is invaluable for investigating the delivery and efficacy of anti-malarial drugs that work by preventing hemozoin formation. In one study, Wood *et al.*[82] obtained resonant Raman spectra of hemozoin while encapsulated within a food vacuole; and in the other, they acquired tip-enhanced Raman scattering (TERS) spectra[137]. While the former study yielded reasonable Raman spectra of parasites at the late trophozoite stage, the sensitivity to hemozoin in resonant Raman spectroscopy is limited because of the intrinsically weak Raman signal especially for parasites in the ring stage. In the latter study, TERS achieved 20-nm spatial resolution in Raman probing that enabled the correlation between Raman bands and the probed position on the hemozoin crystal

(verified by atomic force microscopy). This enabled access to molecular information within a sectioned single cell in the nanoscale range.

Alternatively, attaining SERS from within cells has been demonstrated by the use of gold intracellularly grown nanoparticles (IGN) in mammalian cells.[138-140] Unlike TERS, this approach does not necessitate highly precise TERS equipment for locating and probing cells. Moreover, endosomal/lysosomal pathways and dependence on endocytosis are preferably avoided.[138] Method 2 reported in the Chapter 5 is similar to this method in that silver nanoparticles were directly synthesized in malaria parasites, which dramatically improved the sensitivity to hemozoin and made it possible to study single parasites. However, there was no method available to identify the source of the measured SERS spectrum, especially in case of single parasites, which would limit the use of this method in scientific research.

We would like to fill this gap by reporting a procedure that can identify the source of the SERS signal down to the level of single parasites in the ring stage by comparing the SERS spectra measured from isolated hemozoin with those from single parasites. This procedure is used in combination with the ultra-sensitive SERS method reported in the previous chapter to demonstrate for the first time that hemozoin from single parasites in the ring stage can be detected using SERS according to our best knowledge.

6.2 Methodology

6.2.1 Parasite culture

The use of human blood strictly followed protocols and guidelines that were approved by the domain-specific review board of Nanyang Technological University (IRB number: NTU-IRB 11/12/2011). Blood component collection service was provided by Blood Transfusion Service and Blood Donation Centre of National University Hospital. All individuals gave informed consent.

6.2.2 *Hemozoin isolation*

Supernatant in the parasite culture medium after 5 cycles of parasite growth was collected and spin down by centrifuging at 5000 rpm for 5 min (Sartorius 2-16, Sigma Laborzentrifugen, Ostrode, Germany) to collect floating hemozoin. The hemozoin sediment was then washed by RPMI 1640 (RPMI 1640 Medium, Life Technologies, Grand Island, USA) to remove cell debris such as lipid and protein.

6.2.3 *Red blood cell lysis*

A total of 10- μ l infected blood sample were dispersed in 50-ml deionized water and sonicated for 5 min. Released parasites were collected by centrifuging the sample at 5000 rpm for 5 min (Sartorius 2-16, Sigma Laborzentrifugen, Ostrode, Germany) and resuspended in 1-ml PBS.

6.2.4 *Synthesis of silver nanoparticles and preparation before Raman measurements*

Here we synthesized the silver nanoparticles using the reduction method [28, 116, 120, 141]. A total of 5-ml hydroxylamine hydrochloride (0.03 mM, MP Biomedicals, Santa Ana, USA) and NaOH (0.15 mM) was mixed with 33- μ l Triton X-100 (Triton X-

100 Detergent, Bio-Rad Laboratories, Hercules, USA). The mixture was then added into 45-ml aqueous AgNO_3 (Silver nitrate, Merck, Kenilworth, USA) with a concentration of 1.11 mM, drop-wise over a period of 8 min. The resulting solution was then sonicated (Elma E30H, Elma, Wetzikon, Switzerland) for 30 min. The generated silver nanoparticles was centrifuged and resuspended in 5-ml solution. The suspension was sonicated for 5 min for later use.

In hemozoin measurements, the silver nanoparticle suspension was mixed with hemozoin suspension isolated earlier (1:1, v/v) in ultrasonic bath for 2 min. During Raman measurements, the mixture was smeared on a glass slide covered by aluminum foil as the substrate and dried at room temperature.

In lysed cell measurements, the silver nanoparticle suspension was mixed with lysed cell suspension (1:1, v/v). The mixture was smeared on a glass slide covered by aluminum foil as the substrate for Raman measurements.

6.2.5 Synthesis of silver nanoparticles (NPs) inside parasites

In this step, silver nanoparticles were synthesized inside parasites directly, which can achieve closer contact with hemozoin at a higher concentration than the alternative method[142] in which nanoparticles synthesized separately are mixed with lysed parasites together. It should be highlighted that this method keeps the locally high hemozoin concentration in parasites because it intentionally avoids lysing parasites. Firstly, RBCs lysis was conducted as described earlier. A total of 10- μl lysed blood was dispersed in 50-ml deionized water and sonicated for 10 min to achieve blood cell lysis

without lysing parasites. The resulting suspension was centrifuged at 5000 rpm and resuspended in 4.5-ml AgNO_3 (Silver nitrate, Merck, Kenilworth, USA) solution at a concentration of 1.11 mM. Then 33- μl Triton X-100 (Triton X-100 Detergent, Bio-Rad Laboratories, Hercules, USA) was mixed with 5-ml solution of hydroxylamine hydrochloride (0.03 mM, MP Biomedicals, Santa Ana, USA) and NaOH (0.15 mM). A total of 0.5 ml of the mixture was added drop-wise over a period of 8 min to the aqueous AgNO_3 to reduce silver. In this step, the volume of Triton X-100 was low and it mostly bonded to silver nanoparticles to prevent them from aggregation, which limited its effect on cell lysis to a low level. The resulting solution was then sonicated (Elma E30H, Elma, Wetzikon, Switzerland) for 30 min. Finally, the sonicated solution was smeared on a glass slide covered by aluminum foil as the substrate for Raman measurements.

6.2.6 Raman measurements and data processing

A micro-Raman spectrometer system (inVia, Renishaw, Aberdeen, UK) coupled with a microscope (Alpha 300, WITec, Ulm, Germany) in a backscattering geometry was used to measure all Raman spectra. The system was coupled to a Czerny-Turner type spectrograph ($f = 250$ mm) with a holographic grating (1800 grooves/mm) and a RenCam CCD detector (inVia, Renishaw, Aberdeen, UK), which yielded a spectral resolution of 2 cm^{-1} . A 633-nm diode laser (Renishaw, Aberdeen, UK) was used for excitation with a focal spot size about 2-3 μm on the sample through a microscope objective lens (50X, NA=0.6, Leica, Wetzlar, Germany). The exposure time was 10 seconds and the excitation was 0.5 mW for all SERS measurements. The combination of similar exposure time and excitation power was also used previously in our magnetic

enriched SERS study[28], which can achieve the balance between the maximization of SERS intensity and the minimization of the thermal effect. Since the power of excitation laser was low and the laser beam was focused inside the center of cells instead of cell membrane, the cell would not be damaged during the Raman measurements as confirmed by the comparison in cell morphology before and after the measurements.

In an infected blood sample with parasites in the schizont stage as shown in Fig. 6.1, the Raman spectrum was acquired from dark spots inside the cells, which often represent aggregated hemozoin, based on the white light image. During the measurements of normal blood and infected blood in the ring stage, the laser was focused at the center of an RBC. Similarly, during the measurements of hemozoin, parasites mixed with silver nanoparticles and parasites with silver NPs inside as shown in Fig. 6.2, the Raman spectra were measured from dark spots around 2-3 μm in diameter, which were comparable in size to parasites in the ring stage. Such Raman measurements were repeated in five different RBCs during RBC measurements or five different dark spots during hemozoin or parasite measurements in every sample and averaged to yield the mean spectrum and calculate the standard deviation at each wavenumber. Then the standard deviations at all wavenumbers were used to create the shadow area in Fig. 6.1 and Fig. 6.2, in which the height of the shadow above or below the spectrum is exactly one standard deviation at every corresponding wavenumber. In each raw spectrum, a fifth-order polynomial was used to fit the fluorescence background and then the polynomial was subtracted from the raw spectrum to generate

the clean Raman spectrum.

6.2.7 Procedure of sample postprocessing to confirm the existence of parasites in the ring stage at the focal location

It should be noted that infected RBCs with parasites in the ring stage cannot be differentiated from normal RBCs based on white light images in the process of acquiring data for Fig. 6.1. Similarly, aggregated silver NPs and parasites with NPs inside looked alike in the process of acquiring data for Fig. 6.2. To confirm that a SERS spectrum was truly measured from an infected RBC or parasites in the ring stage, Giemsa staining was performed on every sample slide (after SERS spectra acquisition) following methanol fixation as described below. After Raman measurements, the sample slide was first immersed in methanol solution (Methanol AnalaR Normapur, VWR International, Singapore) for 10 s and then taken out to dry in air. Then the sample slide was placed back to the same location on the microscope stage so that the region of white light imaging was identical to that in Raman measurement. White light images were taken using the built-in microscope in the Raman system to look for purples rings that are the sign of malaria infection.

6.2.8 Ethics statement

The use of human blood strictly followed protocols and guidelines that were approved by the domain-specific review board of Nanyang Technological University (IRB number: NTU-IRB 11/12/2011). Blood component collection service was provided by Blood Transfusion Service and Blood Donation Centre of National University Hospital. All individuals gave informed consent.

6.3 Results

(b)

(c)

Figure 6.1 (a) Spontaneous Raman spectra of hemozoin isolated from infected blood samples, infected RBCs in the schizont stage, infected RBCs in the ring stage and

normal blood. The original amplitudes of all spectra are maintained to facilitate comparison. (b) A white light microscopic image of infected blood that contains infected RBCs in the schizont stage and the ring stage as well as normal RBCs. (c) A Giemsa stained image taken from the same region in (b). An infected RBC in the schizont stage, an infected RBC in the ring stage and a normal RBC are indicated, respectively, by the thin solid arrow (red), thick solid arrow (blue) and thin dashed arrow (green) in (b) and (c).

Figure 6.1 compares the spontaneous Raman spectra of hemozoin isolated from infected blood samples, infected RBCs in the schizont stage, infected RBCs in the ring stage and normal blood. Most prominent vibrational features of hemozoin such as ν_{15} at 754 cm^{-1} , ν_{22} at 1120 cm^{-1} , ν_{11} at 1551 cm^{-1} , ν_2 at 1570 cm^{-1} , and ν_{10} at 1628 cm^{-1} are shown in the spectra of hemozoin and infected RBCs, but not in the spectra of normal RBCs. The locations of these peaks are consistent with those reported Raman peaks of hemozoin [82, 143]. It should be noted that the fluorescence background in the raw spectra of infected RBCs is much higher than the isolated hemozoin, due to hemoglobin and other cellular components. In addition, the Raman peak of hemoglobin at 666 cm^{-1} shows up in the spectra of RBCs, but not in hemozoin spectra. By comparing the spectrum between from hemozoin and normal RBCs, we can find that normal RBCs share some prominent peaks with hemozoin, such as 1120 cm^{-1} and 1570 cm^{-1} , and contribute to some peaks close to the hemozoin peak such as 748 cm^{-1} . These shared peaks in normal blood could be attributed to hemoglobin in the cells. Figure 6.1(b) shows a microscopic image (100X Objective lens, Olympus BX53) of an

infected blood sample. One can easily differentiate the infected RBC in the schizont stage (indicated by the thin solid arrow) from the normal RBC (indicated by the thin dashed arrow). However, based on the morphology of cells in Fig. 6.1(b), one can hardly differentiate between the infected RBC in the ring stage (indicated by the thick solid arrow) and the normal RBC. It became even more difficult using a common Raman microscope with a 50X objective lens as in this study. Figure 6.1(c) shows the Giemsa stained image taken from the same region as in Fig. 6.1(b) to confirm the status of RBCs and the stage of parasites. By comparing Fig. 6.1(b) and Fig. 6.1(c), it is straightforward to observe that Giemsa staining makes it easier to recognize infected blood cells even with parasites in the ring stage.

Figure 6.2 (a) SERS spectra of hemozoin, parasites (in the ring stage) with silver nanoparticles synthesized inside and parasites (in the ring stage) mixed with nanoparticles. The original amplitudes of all spectra are maintained to facilitate comparison. (b) Giemsa stained image of parasites with nanoparticles synthesized inside from the center of which Raman measurements were taken. (c) Giemsa stained image of parasites mixed with nanoparticles from the center of which Raman measurements were taken.

Figure 6.2 depicts the SERS spectra of hemozoin mixed with silver nanoparticles, intact parasites (in the ring stage) with silver nanoparticles synthesized inside and intact parasites (in the ring stage) mixed with silver nanoparticles. Comparing spectral features between hemozoin and intact parasites with silver nanoparticles inside, it can be found that most bands corresponding to hemozoin are identical, including ν_{15} at 754 cm^{-1} , ν_{22} at 1120 cm^{-1} , ν_{11} at 1551 cm^{-1} , ν_2 at 1570 cm^{-1} and ν_{10} at 1628 cm^{-1} . The difference should be also noted that ν_{11} at 1551 cm^{-1} in the spectrum of parasites with nanoparticles inside can be hardly observed while ν_2 at 1570 cm^{-1} appears as the major band. This observation could be attributed to the influence of hemoglobin. On the other hand, because silver nanoparticles hardly have any chance to interact with hemozoin inside parasites, intact parasites mixed with silver nanoparticles show a much weaker Raman signal compared with the other two. The peak intensities are so low that some peaks cannot be observed since their intensities are close to the noise level. Fig. 6.2(b) shows the Giemsa stained image of the focal location of a sample with parasites in which NPs were synthesized inside, which was taken after Raman measurements with sample postprocessing by methanol fixation followed by Giemsa staining. It can be seen that the dark region at the center was covered mostly by nanoparticles but the region was also stained partially in purple, which confirms the existence of parasites. Fig. 6.2(c) demonstrates the focal location of a sample in which parasites were mixed with NPs after Giemsa staining following the same procedure. The purple ring at the center indicates a parasite in the ring stage at the focus of Raman measurement.

6.4 Discussion

Without a concentrating step, the detection of hemozoin in single parasites remains a key challenge. The extremely low hemozoin amount in parasites in the ring stage, which is an early stage in the life cycle of malaria parasites, makes it even more difficult. Here we present the data that demonstrate the feasibility and advantage of surface enhanced Raman spectroscopy for detecting hemozoin from single parasites in the ring stage for the first time. According to Fig. 6.1, spontaneous Raman spectroscopy can be used to identify infected RBCs with parasites in the late stage such as the schizont stage out of normal RBCs but cannot differentiate between infected RBCs with parasites in the ring stage and normal RBCs. The variations of measurements from hemozoin could be attributed to the inhomogeneous distribution of hemozoin crystals, while the variation of infected RBCs in this study could be due to the asynchronization of parasites in the infected sample within the ring stage. Although the synchronization procedure has been performed to synchronize parasites in the ring stage, parasites could have an uneven distribution from the early ring stage to the late ring stage.

SERS requires nanoparticles to contact with hemozoin crystals, which cannot be achieved by simply mixing a parasite with SERS active nanoparticles, as shown by the low magnitude for the SERS spectra from parasites mixed with nanoparticles in Fig. 6.2, because hemozoin would stay inside the parasite without parasite lysis. Only the spectrum measured from parasites with silver nanoparticles synthesized inside show significant agreement with the Raman spectrum of hemozoin, which suggests that hemozoin was measured from single parasites. One source of variation in the SERS

measurements of parasites could be the asynchronization of parasites within the ring stage, which is the same factor as in the discussion about Fig. 6.1. Moreover, the size variation in nanoparticles synthesized inside parasites, which affected the enhancement factor of SERS, acts as another source for the variation in the spectra measured from parasites with silver NPs synthesized inside.

Malaria parasites convert free heme to hemozoin to avoid the toxicity of heme to themselves[144]. Therefore the prevention of hemozoin formation is the target for quinolone and most other malaria drugs[145]. Some anti-malaria drugs including artemisinin, aryl-alcohol and peroxides, work by entering the vacuoles of parasites and interacting with heme. To monitor the effect of anti-malaria drugs in early treatment, it requires the detection or monitoring of hemozoin formation in early-stage parasites. Compared to the previously reported micro-Raman based detection[82] and tip-enhanced Raman scattering for the detection of hemozoin inside parasites[137], our SERS strategy provides higher enhancement to achieve the detection of hemozoin in parasites in the ring stage with a simpler procedure of nanoparticle preparation and lower cost. Moreover, our approach in the synthesis of silver nanoparticles inside parasites can be also used to enhance the Raman signal of other chemicals including anti-malarial drugs. The drug-heme bonding can be monitored by directly measuring the Raman signal of the drug inside the parasites or detecting the changes in the Raman signal of hemozoin. The decrease in the relevant Raman peaks of hemozoin may indicate the effectiveness in drug treatment. The simplicity and high signal enhancement of our SERS based technique infer its great potential in malaria diagnosis

and malarial drug development.

Despite the rapid development of SERS based methods for malaria diagnosis as surveyed earlier, there are still a few major disadvantages to be addressed, which include the requirement of hot spot search and/or long time required in the hot spot searching as well as the high cost of Raman systems. Several potential strategies could be used to overcome these disadvantages. There have been efforts to enhance SERS sensitivity to waive the requirement of hot spot search[146] in which SERS measurements at random locations rather than selected hot spots are taken. This strategy can save time for hot spot search compared to the method of measuring hot spots but may result in greater variation. To achieve the highest SERS sensitivity from hot spot measurements and meanwhile reduce time for hot spot search, one could apply an automated hot-spot searching method[132]. With a proper configuration in the method, one can scan the entire sample in a reasonable period of time and “hot spots” will be selected automatically. The disadvantage in the high cost of a commercial Raman spectrometer with decent spectral resolution could be overcome by utilizing a cost effective spectrometer[135] to measure Raman spectra with a low spectral resolution then reconstructing the corresponding Raman spectra with a high spectral resolution using a spectral recovery method we have developed recently[107]. Moreover, most steps for blood sample preparation in our method can be implemented in a microfluidic chip[134] to enable automatic and standardized sample preparation, which could further increase the possibility of deploying SERS in the field.

6.5 Conclusion

Our study in using SERS for hemozoin detection in single parasites of the ring stage offers a promising strategy to investigate the mechanism of heme metabolism in malaria infection and a tool to evaluate the effectiveness of anti-malaria drugs. The method of synthesizing nanoparticles inside parasites could be also used to improve SERS sensitivity for early malaria diagnosis as demonstrated in the previous chapter.

Chapter 7: Conclusions and Future Directions

7.1 Conclusions

This dissertation presents a series of studies on the development of Raman spectroscopy and surface enhanced Raman spectroscopy techniques for biomedical applications including neural stem cell detection, corneal infection diagnosis, early malaria diagnosis and single malaria parasite detection. Spontaneous Raman spectroscopy was demonstrated feasible for neural stem cell detection and discrimination from their lineages. The potential of Raman spectroscopy in corneal infection diagnosis was also investigated. Novel SERS methods were developed to improve the Raman signal of malaria parasite and thus achieve ultrasensitive detection and even single parasite detection. The main findings of these studies are summarized as follows.

First, Raman spectroscopy can be used to discriminate neural stem cells from their lineages. In Chapter 3, we applied spontaneous Raman spectroscopy to the characterization of neural stem cells and their lineages. The employment of the PCA-LDA method on the analysis of the Raman data yielded high accuracy in cell classification. Moreover, we evaluated the influence of system parameters during spectral acquisition on the quality of measured Raman spectra, which yielded a set of optimal system parameters facilitating future studies.

Second, Raman spectroscopy can be utilized to detect fungi infected cornea. In Chapter 4, we initially demonstrated that the fungi of different species can be differentiated from each other by their Raman spectra. And then both infected corneas

and healthy corneas were measured with the same configuration, which showed significant difference in spectral features. Therefore, the results suggested the great potential of Raman spectroscopy for corneal infection diagnosis.

Third, SERS techniques are useful for both quantitative sample analysis and ultra-sensitive detection in the early stage of malaria infection. In Chapter 5, we introduced two SERS strategies for early malaria detection. In Method 1, silver nanoparticles were synthesized beforehand and then mixed with hemozoin isolated from malaria infected blood. SERS spectra were captured from the mixture. Method 1 provided the straightforward linear correlation between spectral intensity and hemozoin contribution in a large range, while the sensitivity was comparable to commercial techniques such as QBC and RDT. In Method 2, silver nanoparticles were synthesized directly inside the malaria parasites to achieve close contact with hemozoin inside. The sensitivity of the second method was slightly better than the standard method—Giemsa stained microscopy. These two strategies indicated that SERS techniques have great potential in both ultrasensitive and quantitative measurements of hemozoin for early malaria detection.

Finally, based on the second method developed in Chapter 5, we implemented the ultra-sensitive SERS technique for the detection of hemozoin in single parasites as demonstrated in Chapter 6. Extra silver nanoparticles were filtered away after silver nanoparticles were synthesized inside parasites in the ring stage. SERS spectra were the obtained from intact parasites with silver nanoparticles in them. The existence of parasites in the focal region was confirmed by performing Giemsa stained microscopy

on the sample after Raman measurements. This method offers a promising strategy to investigate the mechanism of heme metabolism in malaria infection and a tool to evaluate the effectiveness of anti-malaria drugs.

7.2 Future Directions

In the series of studies introduced in this dissertation, we primarily focus on Raman spectroscopy and SERS for biomedical applications. More works are required to simplify the system, standardize sample preparation and remove the influence of substrate or impurities. The detailed future directions are described as follows.

In all the studies reported in this dissertation, the performance of our Raman techniques depends on a robust Raman system coupled with a spectrometer. Considering that a commercial Raman system is expensive and bulky, we intend to design cost-effective spectrometer using cheap components. For example, a DVD disk has been used before as a grating and a cell phone can work as the detector and data processor. A laser pointer or another hand-hold laser can be used as the excitation source. Because the excitation power will be low and the throughput will be sub-optimal, the Raman signal obtained with such a cost effective setup will be weaker than that obtained by a commercial system. Additional enhancement method such as SERS or RRS or a spectral reconstruction method may be required for data acquisition or postprocessing. Being cheap and portable, the cost-effective spectrometer will lower down the financial requirement of our Raman and SERS methods and thus broadening its usage in low-resource regions, which is significant especially in the malaria

prevalent region.

Furthermore, since many biochemical components, some of which are undesired, emit Raman signals, it is necessary to study how to appropriately remove the background contribution in measured spectra from undesired Raman sources, for example, the culturing medium in neural stem cell measurements, healthy cornea in infected cornea measurements and hemoglobin in hemozoin measurements of malaria infected blood samples. This problem remains a major challenge in data processing. An automated and rapid background subtraction algorithm to remove influence from already known substrates would play an important role in the Raman measurements of nearly all biomedical samples.

The study in corneal infection diagnosis introduced in Chapter 4 is just started. For the infected cornea group, we only measured *C. a.* infection. In the future, we will continue to research on the corneas infected by other fungi, *A. f.*, *F. s.*, *etc.* The spectra features will be used to predict biological changes during infection. In addition, a reference library that contains the Raman spectra of all common fungal pathogens and cornea infected by these pathogens as well as healthy corneas will be created for quick diagnosis. One concern for transferring Raman techniques for the field use is laser safety. In the present study, the power we used in measurements was 50 mW, which is definitely too high for human eyes. To limit the laser power to below the maximum permissible exposure and protect the eyes, we will develop a special illumination and collection scheme, in which the illuminating laser light will be reflected back instead of passing through the cornea to reach the retina. Moreover, an ultra-sensitive detector

taking advantage of lock-in detection may be needed to collect the weak Raman signal due to power reduction.

In Chapters 5 and 6, we implemented SERS in malaria diagnosis and hemozoin detection in single parasites, respectively. All sample preparation steps were performed in a lab setting, which can be difficult to consistently reproduce in the field. We plan to design a microfluidic chip for simplify sample preparation in the SERS methods. This chip will perform cell lysis, nanoparticle synthesis and sample mixing. The resulting blood sample will be exposed to laser light for Raman measurements. The use of such a chip will further standardize the procedure, thus minimizing the variation caused by the operator and promote the field usage of our SERS methods.

Besides the background removal algorithm and chip development, we can utilize the unique magnetic property of hemozoin to improve the specificity of SERS for hemozoin detection while minimizing the influence of the primary interfering source, i.e. hemoglobin. Due to the difference in magnetic property between hemozoin and hemoglobin, the Raman signal of the two analytes could be affected in different ways under an external magnetic field, which could be utilized to isolate the Raman signal solely contributed by hemozoin. These approaches may further improve the performance of SERS in early malaria diagnosis and other blood borne diseases.

Author publications

Academic Journals

1. K. Chen, C. Yuen, Y. Aniweh, P. Preiser and Q. Liu, Towards ultrasensitive malaria diagnosis using surface enhanced Raman spectroscopy, **Scientific Reports** 6, p. 20177, 2016.
2. K. Chen, C. Perlaki, A. Xiong, P. Preiser, and Q. Liu, Review of surface enhanced Raman spectroscopy for malaria diagnosis and a new approach for detection of single parasites in the ring stage [Invited], **IEEE Journal of Selected Topics in Quantum Electronics** 22, p. 6900509, 2016.

Book Chapter and Conferences Volume

1. K. Chen, Y. H. Ong, C. Yuen and Q. Liu, Surface-enhanced Raman spectroscopy for intradermal measurements, **Imaging in Dermatology**, chap. 13, 1-14, **Elsevier Science**, 2016. doi: 10.1016/B978-0-12-802838-4.00013-3
2. K. Chen, A. Xiong, C. Yuen, P. Preiser and Q. Liu, Investigation of surface enhanced Raman spectroscopy for hemozoin detection in malaria diagnosis, **Proc. SPIE** 9715, p. 97150F, 2016.
3. K. Chen, A. Xiong, C. Yuen, P. Preiser and Q. Liu, Towards field malaria diagnosis using surface enhanced Raman spectroscopy, **Proc. SPIE** 9887, p. 98870M, 2016.

4. K. Chen, A. Xiong, P. Preiser and Q. Liu, Ultrasensitive surface-enhanced Raman spectroscopy for malaria diagnosis, **SPIE Newsrooms**, 2016.

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1. K. Chen, W. Ong, S. Y. Chew and Q. Liu, Parameter optimization in Raman spectroscopy for differentiation of neural progenitor cells and their lineages, **SPIE BiOS**, San Francisco, USA, January 28, 2017.
2. K. Chen, A. Xiong, P. Preiser and Q. Liu, Towards field malaria diagnosis using surface enhanced Raman spectroscopy, **SPIE Photonics Europe**, Brussels, Belgium, April 5, 2016. **(Invited)**
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6. K. Chen, C. Yuen, Y. Aniweh, P. Preiser and Q. Liu, Towards early malaria diagnosis using surface enhanced Raman spectroscopy, *SPIE BIOS*, San Francisco, USA, February 8, 2015.

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