Development of fast spectroscopic imaging techniques for biomedical applications

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**Development of Fast Spectroscopic Imaging
Techniques for Biomedical Applications**

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Development of Fast Spectroscopic Imaging Techniques for Biomedical Applications

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Development of Fast Spectroscopic Imaging Techniques for Biomedical Applications

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At Nanyang Technological University, Singapore

This dissertation presents a series of studies in the development of fast spectroscopic imaging techniques, including diffuse reflectance, fluorescence and Raman spectroscopic imaging, in which the biomedical applications of the techniques are demonstrated.

First, the background of various optical spectroscopy techniques and the current state of art in spectroscopic imaging are presented, in which the new approach of narrow-band measurements followed by spectral reconstruction is introduced.

As a critical step in the new approach, spectral reconstruction based on Wiener estimation have been investigated, which yielded several methods. In particular, the modified Wiener estimation (WE) method was developed to improve the reconstruction accuracy of the diffuse reflectance from narrow-band color measurements. It was demonstrated that the proposed modified WE can reconstruct diffuse reflectance spectra with higher accuracy than the traditional WE method. These diffuse reflectance spectra could be then used to estimate optical properties and further tissue parameters.

Then a sequential weighted WE method was developed to derive tissue parameters

directly from narrow-band color measurements and their ratios without reconstructing diffuse reflectance spectra. It was found that the sequential weighted WE method showed significant improvement in the accuracy of derived tissue parameters compared with the traditional WE method in a phantom experiment. The direct extraction of tissue parameters could facilitate the monitoring of tissue parameters in a large region of interest in real time for clinical diagnosis.

Inspired by the above phantom experiment, narrow-band measurements and their ratios were used for the early prediction of flap occlusion in an animal study. Besides diffuse reflectance imaging, narrow-band autofluorescence imaging was investigated as well. The results showed the high feasibility of using narrow-band imaging to monitor flap occlusion.

We further extend this spectral imaging technique to Raman spectroscopic imaging. The major challenge in Raman imaging is that the Raman signal in biological samples is intrinsically weak, and a Raman spectrum is usually more complex in spectral features such as the number of peaks than diffuse reflectance and fluorescence spectra. Another challenge is that there is no commercial polychromatic camera designed for Raman imaging. We addressed these challenges by applying narrow-band measurements to improve the signal-to-noise ratio and selecting/designing filters for a virtual Raman camera. The results of spectral reconstruction for Raman spectra both with and without fluorescence background showed excellent agreement with measured spectra, which implies the feasibility of using our approach of fast Raman imaging to investigate dynamically changing phenomena in biological samples.

This technique can be also used to recover Raman spectra from low signal-to-noise ratio (SNR) Raman measurements. In this application, a low SNR Raman spectrum is integrated along the wavenumber dimension to reduce the influence of noise, which is followed by spectral reconstruction based on WE to recover the Raman spectrum with high spectral resolution. This approach showed the ability of recovering Raman spectra from measurements with extremely low SNR, which was more accurate than four commonly used de-noising methods.

Despite the many advantages and applications of narrow-band measurements followed by spectral reconstruction, one major limitation of this technique is that a new calibration data is required for each type of samples, which implies a huge burden and may prevent this Raman imaging approach from being widely adopted. To overcome this limitation, we proposed a method to create a universal calibration dataset for spectral reconstruction. Because many biological samples, such as human cells, share the same set of basic biochemical components, the calibration dataset based on these biochemical components will be applicable to all such samples. In this case, only a handful number of Raman measurements are needed to create such a universal calibration dataset. Moreover, the measurements of those basic components can be reused if they are shared by a new category of samples. Therefore, the resources required for creation of the calibration dataset can be dramatically reduced using this new method.

In summary, the proposed spectroscopic imaging technique, which refers to narrow-band measurements followed by spectral reconstruction, is able to realize fast

spectral imaging or the quick extraction of key tissue parameters in the cases of diffuse reflectance, fluorescence and Raman spectroscopy or improve the signal-to-noise ratio of optical spectra from low-SNR measurements. The principle component (PC) based filters applied in the step of narrow-band measurements are found to generate the best performance. A new method has been developed to reduce the resources required for the creation of the calibration dataset in the step of spectral reconstruction. These techniques will be further refined and explored to observe fast changing phenomenon in biomedical applications.

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Abstract

This dissertation presents a series of studies in the development of fast spectroscopic imaging techniques, including diffuse reflectance, fluorescence and Raman spectroscopic imaging, in which the biomedical applications of the techniques are demonstrated.

First, the background of various optical spectroscopy techniques and the current state of art in spectroscopic imaging are presented, in which the new approach of narrow-band measurements followed by spectral reconstruction is introduced.

As a critical step in the new approach, spectral reconstruction based on Wiener estimation have been investigated, which yielded several methods. In particular, the modified Wiener estimation (WE) method was developed to improve the reconstruction accuracy of the diffuse reflectance from narrow-band color measurements. It was demonstrated that the proposed modified WE can reconstruct diffuse reflectance spectra with higher accuracy than the traditional WE method. These diffuse reflectance spectra could be then used to estimate optical properties and further tissue parameters.

Then a sequential weighted WE method was developed to derive tissue parameters directly from narrow-band color measurements and their ratios without reconstructing diffuse reflectance spectra. It was found that the sequential weighted WE method showed significant improvement in the accuracy of derived tissue parameters compared with the traditional WE method in a phantom experiment. The direct extraction of tissue parameters could facilitate the monitoring of tissue parameters in

a large region of interest in real time for clinical diagnosis.

Inspired by the above phantom experiment, narrow-band measurements and their ratios were used for the early prediction of flap occlusion in an animal study. Besides diffuse reflectance imaging, narrow-band autofluorescence imaging was investigated as well. The results showed the high feasibility of using narrow-band imaging to monitor flap occlusion.

We further extend this spectral imaging technique to Raman spectroscopic imaging. The major challenge in Raman imaging is that the Raman signal in biological samples is intrinsically weak, and a Raman spectrum is usually more complex in spectral features such as the number of peaks than diffuse reflectance and fluorescence spectra. Another challenge is that there is no commercial polychromatic camera designed for Raman imaging. We addressed these challenges by applying narrow-band measurements to improve the signal-to-noise ratio and selecting/designing filters for a virtual Raman camera. The results of spectral reconstruction for Raman spectra both with and without fluorescence background showed excellent agreement with measured spectra, which implies the feasibility of using our approach of fast Raman imaging to investigate dynamically changing phenomena in biological samples.

This technique can be also used to recover Raman spectra from low signal-to-noise ratio (SNR) Raman measurements. In this application, a low SNR Raman spectrum is integrated along the wavenumber dimension to reduce the influence of noise, which is followed by spectral reconstruction based on WE to recover the Raman spectrum with high spectral resolution. This approach showed the ability of recovering Raman

spectra from measurements with extremely low SNR, which was more accurate than four commonly used de-noising methods.

Despite the many advantages and applications of narrow-band measurements followed by spectral reconstruction, one major limitation of this technique is that a new calibration data is required for each type of samples, which implies a huge burden and may prevent this Raman imaging approach from being widely adopted. To overcome this limitation, we proposed a method to create a universal calibration dataset for spectral reconstruction. Because many biological samples, such as human cells, share the same set of basic biochemical components, the calibration dataset based on these biochemical components will be applicable to all such samples. In this case, only a handful number of Raman measurements are needed to create such a universal calibration dataset. Moreover, the measurements of those basic components can be reused if they are shared by a new category of samples. Therefore, the resources required for creation of the calibration dataset can be dramatically reduced using this new method.

In summary, the proposed spectroscopic imaging technique, which refers to narrow-band measurements followed by spectral reconstruction, is able to realize fast spectral imaging or the quick extraction of key tissue parameters in the cases of diffuse reflectance, fluorescence and Raman spectroscopy or improve the signal-to-noise ratio of optical spectra from low-SNR measurements. The principle component (PC) based filters applied in the step of narrow-band measurements are found to generate the best performance. A new method has been developed to reduce

the resources required for the creation of the calibration dataset in the step of spectral reconstruction. These techniques will be further refined and explored to observe fast changing phenomenon in biomedical applications.

Chapter 1 Overview

This dissertation presents a series of studies in the development of fast spectroscopic imaging techniques, including diffuse reflectance, fluorescence and Raman spectroscopic imaging, in which the biomedical applications of the techniques are demonstrated. The following chapters are arranged in a logical order by which the significance of spectroscopic imaging, the shortcomings of the current spectroscopic imaging techniques, our new spectroscopic imaging techniques and their applications are described. However, each chapter can also be read independently.

Chapter 2 introduces the background of various optical spectroscopy techniques, i.e. the principle of the diffuse reflectance spectroscopy, fluorescence spectroscopy and Raman spectroscopy. Due to the low data acquisition speed, spectroscopic imaging techniques are developed and the current state of art in spectroscopic imaging is described. In addition, new approach of narrow-band measurements followed by spectral reconstruction is introduced, which is the fundamental of our new techniques.

In Chapter 3, the modified Wiener estimation (WE) method was developed to improve the reconstruction accuracy of the diffuse reflectance from narrow-band color measurements. It was demonstrated that the proposed modified WE can reconstruct diffuse reflectance spectra with higher accuracy than the traditional WE method. With the help of the proposed modified WE, the reconstructed diffuse reflectance with higher accuracy could provide more accurate optical properties and subsequently more accurate tissue parameters for diagnosis.

In Chapter 4, based on the feasibility verified in Chapter 3, a sequential weighted WE

method was developed to derive tissue parameters directly from narrow-band color measurements and their ratios without reconstructing diffuse reflectance spectra. It was found that the sequential weighted WE method showed significant improvement in the accuracy of derived tissue parameters compared with the traditional WE method in a phantom experiment. The direct extraction of tissue parameters could facilitate the monitoring of tissue parameters in a large region of interest in real time for clinical diagnosis. In addition, a method to correct the instrument to instrument difference is proposed, in which the calibration data measured by another system can still be used.

Inspired by the above phantom experiment in Chapter 4, narrow-band measurements and their ratios were used for the early prediction of flap occlusion in an animal study in Chapter 5. Besides diffuse reflectance imaging, narrow-band autofluorescence imaging was investigated as well. The results showed the high feasibility of using narrow-band imaging to monitor flap occlusion.

In Chapter 6, we further extend this spectral imaging technique to Raman spectroscopic imaging. The major challenge in Raman imaging is that the Raman signal in biological samples is intrinsically weak, and a Raman spectrum is usually more complex in spectral features such as the number of peaks than diffuse reflectance and fluorescence spectra. Another challenge is that there is no commercial polychromatic camera designed for Raman imaging. We addressed these challenges by applying narrow-band measurements to improve the signal-to-noise ratio and selecting/designing filters for a virtual Raman camera. The results of spectral

reconstruction for Raman spectra both with and without fluorescence background showed excellent agreement with measured spectra, thus the proposed spectroscopic imaging techniques represented a new direction in fast Raman imaging for the investigation of fast changing phenomena.

In Chapter 7, this technique is extended to recover Raman spectra from low signal-to-noise ratio (SNR) Raman measurements. In this application, a low SNR Raman spectrum is integrated along the wavenumber dimension to reduce the influence of noise, which is followed by spectral reconstruction based on WE to recover the Raman spectrum with high spectral resolution. This approach showed the ability of recovering Raman spectra from measurements with extremely low SNR, which was more accurate than four commonly used de-noising methods. The results also prove the feasibility of the Raman imaging from sample with low signal to noise ratio Raman spectrum, especially for those biological samples.

In Chapter 8, a method to create a universal calibration dataset for spectral reconstruction is proposed. Despite the many advantages and applications of narrow-band measurements followed by spectral reconstruction, one major limitation of this technique is that a new calibration data is required for each type of samples, which implies a huge burden and may prevent this Raman imaging approach from being widely adopted. To overcome this limitation, we proposed a method to create a universal calibration dataset for spectral reconstruction. Because many biological samples, such as human cells, share the same set of basic biochemical components, the calibration dataset based on these biochemical components will be applicable to

all such samples. In this case, only a handful number of Raman measurements are needed to create such a universal calibration dataset. Moreover, the measurements of those basic components can be reused if they are shared by a new category of samples. Therefore, the resources required for creation of the calibration dataset can be dramatically reduced using this new method.

In Chapter 9, we summarize and propose the future direction of those spectroscopic imaging techniques, including diffuse reflectance, fluorescence and Raman imaging. Further refinement of these techniques would be helpful for the application of optical spectroscopic techniques to observe fast changing phenomenon in biomedical applications.

Chapter 2: Introduction

2.1 Skin

The skin is a complex, multilayered organ, which produce several specialized derivative structure called appendages and consists of heterogeneous cells types and extracellular components [1]. It usually consists of three layers: epidermis, dermis and hypodermis. The detail structure of human skin is shown in Fig. 2.1.

Fig. 2. 1 Anatomy of the human skin. Figure adapted from reference [2]

The color of skin is mainly contributed by the absorption and scattering properties of the skin. The hemoglobin and melanin are the dominant absorbers, while the primary scatterer is the filamentous protein and further scatter is attributed to the cell nuclei, cell walls, etc. The absorption refers to the reduction in the light intensity and scattering describes a change in the direction, polarization or phase of light. Those are

important biomarkers, thus the color of the skin has long been used as a subjective adjunct to the detection and diagnosis of disease [3]. Therefore, the optical techniques, such as optical spectroscopy that can provide much more information than color measurements, show great potential for the disease diagnosis.

2.2 Background in Optical Spectroscopy for Tissue Characterization

Optical spectroscopy for tissue characterization takes advantage of the interactions between light and tissue components, which includes light absorption, light scattering and fluorescence. Due to the high sensitivity and non-invasiveness of optical techniques and the rapid progress in the development of small light sources, sensitive detectors and fiber optic technology, optical spectroscopy has achieved tremendous success in many biomedical applications. Since this dissertation is focused on three common optical spectroscopy techniques, including diffuse reflectance spectroscopy, fluorescence spectroscopy, Raman spectroscopy, the principles of using these techniques for tissue characterization, particular applications and limitations will be reviewed in the following paragraphs.

2.2.1 Diffuse Reflectance Spectroscopy

Diffuse reflectance spectroscopy measures light reflected from the surface after traveling inside a sample [4], in which the primary sources of optical contrast are light absorption and scattering. Since both the absorption and scattering properties of a tissue vary with wavelength, the measured diffuse reflectance spectrum can be used to estimate the absorption and the scattering properties of a tissue [5]. The absorption

coefficient is related to the concentrations of physiological absorbers in tissues, such as hemoglobin and melanin. The scattering properties are related to the shape, size and density of tissue scatterers. Diffuse reflectance spectroscopy has been explored various biomedical and scientific applications [6-9].

The previous studies have shown that optical properties can be estimated by using both analytical and numerical models. In general, these methods can be categorized into diffusion theory based methods [10], Monte Carlo based inverse methods [11] and empirical methods [12]. In a diffusion theory based method, a model of light transport based on diffusion theory is established, which relates measured diffuse reflectance with the reduced scattering coefficient and absorption coefficient. Then a least-square regression is often utilized to fit measured data to the diffusion theory based model to minimize the difference between modeled diffuse reflectance spectra and measured diffuse reflectance spectra. However, this technique only works well for samples with low absorption and strong scattering. In Monte Carlo based inverse methods, a forward Monte Carlo model is often used to simulate diffuse reflectance for given absorption coefficient and reduced scattering coefficient. By using the non-linear least-square optimization, the values of absorption coefficient and reduced scattering coefficient are modified iteratively until an acceptable difference between the measured diffuse reflectance value and simulated value is achieved. Although this method is accurate, it requires massive computation. In an empirical method, the empirical model for extraction of optical properties is developed according to experimental or simulated results. For example, a lookup table based model [13] is

built using diffuse reflectance measurements from tissue phantoms with known scattering and absorption coefficients. Therefore, the limitation of this method is that it relies heavily on experiments or simulations and calibration is often required.

Fig. 2. 2 Schematic of an optical system for diffuse reflectance spectroscopy.

The typical setup for diffuse reflectance spectroscopy is shown in Fig. 2.2, which consists of three basic components, i.e. a broad-band light source for illumination, a spectrometer for detection and a fiber-optic probe for light delivery and collection. Due to the employment of the fiber optic-probe, the measurement can be only performed point by point. So it is time consuming to collect spectral information from a large tissue area with high spatial resolution. Slow data acquisition prohibits the use of diffuse reflectance spectroscopy in those applications in which the observation of fast changing phenomena in a large tissue area with high spatial resolution is required. In such applications, only spectral imaging techniques could potentially fulfill the requirement.

2.2.2 Principle of Fluorescence Spectroscopy

Fluorescence spectroscopy is based on the emission of fluorescence light from

fluorophore molecules upon excitation. A fluorophore molecule is first excited from the electronic ground state (S_0) to an excited state (S_1) and then it relaxes back to the ground state. The relaxation process generates energy in the form of fluorescence emission. The energy diagram of fluorescence emission is shown in Fig. 2.3. Due to the energy loss from the excitation and emission, the emitted light usually has longer wavelengths compared with the excitation light.

Because of rich endogenous fluorophores, autofluorescence spectroscopy can provide biochemical and morphological changes non-invasively in tissues. The common endogenous fluorophores include amino acids (tryptophan, tyrosine and etc.) [14, 15], structural proteins (collagen and elastin)[16, 17], FAD (flavin adenine dinucleotide) and NADH (reduced nicotinamide dinucleotide) [18], vitamins [19, 20] and porphyrins [21]. In addition, fluorescence spectroscopy could provide quantitative information about these fluorophores provided that the effects of light absorption and scattering have been eliminated, because fluorescence contribution of each fluorophore is generally proportional to its concentration at low concentrations. However, the fluorescence spectra of multiple fluorophores are highly overlapping, which can induce errors in the processing spectral decomposition. The data acquisition speed is also slow when a large field of view with high spectral resolution is needed, thus a fast spectral imaging technique is highly demanded.

Fig. 2. 3 Schematic of fluorescence emission. Figure adapted from reference [22]

Different from a diffuse reflectance spectroscopy system, a fluorescence spectroscopy system uses a light source with a narrower spectral band, such as a light-emitting diode (LED) or laser, and a long pass filter is usually employed before the spectrometer to filter out the excitation light, as shown in Fig. 2.4.

Fig. 2. 4 Schematic of an optical system for fluorescence spectroscopy.

2.1.3 Principle of Raman Spectroscopy

Raman spectroscopy is a spectroscopic technique that measures the inelastic scattering of photons induced by interaction with molecular bonds [23]. Due to vibrational energy loss or gain in molecules, Raman radiation is shifted in frequency relative to the excitation light [24]. The frequency shift in Raman scattering is directly related to the vibrational, rotational or other low-frequency states of molecular bonds[25]. Thus Raman spectra contain rich biochemical information. For example,

useful biochemical information about the composition of the sample and the concentration or amount of each component can be extracted from Raman spectra, because the intensity of Raman peaks in solution is in general proportional to the concentration of particular species [26]. What's more, Raman spectroscopy is usually a non-destructive and label-free technique [27], which is particularly suitable for biomedical applications. Because of these advantages, Raman spectroscopy has been widely used in biomedical applications recently [28, 29]. However, one disadvantage of Raman spectroscopy is that the Raman signal is very weak, especially in those biological samples. The Raman scattering intensity is typically 10^{-9} to 10^{-6} of the intensity of the Rayleigh background [23]. Thus, long integration time is always needed and Raman data acquisition is generally slow, which prohibits Raman spectroscopy from being used to investigate fast changing phenomena especially in biological samples.

The schematic of the Raman spectroscopy system is very similar to the fluorescence spectroscopy system, as shown in Fig. 2.4. However, Different from fluorescence spectroscopy, Raman spectroscopy requires a more intense monochromatic light source for excitation such as a laser and a more sensitive detector.

2.2 State of Art in Spectral Imaging Techniques

Several different fast spectral imaging techniques, e.g. point-scanning, line scanning, multispectral imaging, snapshot spectroscopic imaging, spectral imaging reconstructed from narrow-band/wide-band measurements, have been developed to

overcome the limitation of slow data acquisition in the traditional optical spectroscopy. The following sections will give a brief introduction to the currently available spectral imaging techniques.

2.2.1 Point Scanning

The point scanning method collects the spectral information point by point by using rotating mirrors[30]. As shown in Fig. 2.5, a single point source is scanned along the x and y dimensions and then redirected to the dispersion devices by rotating mirrors. After passing through the dispersion devices, the spectral information is recorded by a linear array detector. Although both spectral and spatial information can be collected, the point scanning mode is always time-consuming when a high spatial resolution is required because of numerous scans in both x and y dimensions.

Fig. 2. 5 Schematic of point scanning method

2.2.2 Line Scanning

Different from the point scanning method, the line scanning method[31, 32] is able to acquire both spectral and spatial information simultaneously in a slit area, as shown in Fig. 2.6. Illuminated light is shaped into a line using cylindrical optics or a scanning mechanism and Raman spectra are collected by a two-dimensional detector array, e.g. charge-coupled device (CCD). While spatial information is acquired along the illumination line, the spectral information is collected in the dimension perpendicular to the illumination line. Therefore, only one-dimensional scan is needed to get the spectral imaging information and the data acquisition speed is improved significantly. However, this method causes field-curvature artifacts and its actual speed is limited by the requirement of autofocusing prior to data acquisition[33].

Fig. 2. 6 Schematic of line scanning method

2.2.3 Multispectral Imaging

Multispectral imaging refers to the acquisition of spectral information at each pixel of an imaged scene[34]. Multispectral images are acquired by an instrument that can

simultaneously record spectral and spatial information of a sample [35]. It captures image data at certain wavelengths by specific filters or instruments that are sensitive to particular wavelengths. A multispectral imaging system usually consists of a broadband light source, a high-performance CCD camera, a wavelength selection device and a computer for control and data analysis as shown in Fig. 2.7. The dispersive device can be a filter wheel [36] or a tunable filter such as acousto-optic tunable filters [37, 38] and liquid crystal tunable filters [39, 40] in multispectral imaging. Fourier transform (FT) based Michelson interferometer can be also used as a dispersive device [41-44]. The advantage of this technique is that both the spectral and spatial information can be recorded simultaneously [45]. However, the acquisition speed would be slow for multispectral imaging when a high spectral resolution is required because this means a large number of images at different wavelengths will need to be taken. This limitation originates from the employment of the dispersive device, for which the information at only one or a couple of wavelengths can be collected in one measurement.

Fig. 2. 7 Schematic of a multispectral imaging system.

2.2.4 Snapshot Spectroscopic Imaging

Snapshot spectroscopic imaging is a technique in which both the spatial and spectral information simultaneously are acquired in a snap shot [46]. This technique provides much faster acquisition compared to traditional multispectral imaging reviewed in Section 2.2.3. The key idea in snapshot imaging is that hyperspectral images can be captured all at once on a 2D detector array, thus avoiding the scanning process in traditional multispectral imaging. Due to the lack of moving components, snapshot spectral imaging generates images with increased robustness or compactness and without scanning artifacts [47]. Common snapshot spectral imaging techniques can be divided into two categories: through-put-division techniques and full-throughput techniques [48]. In throughput-division techniques, in which filters are used for wavelength selection, e.g. multi-aperture filtered camera [49] and multi-spectral filter array camera [50], the light collection efficiency is similar to that in a scanning system. Full-throughput snapshot techniques, which have no filters, include computed tomographic imaging spectrometry (CTIS) [51], fiber-reformatting imaging spectrometry (FRIS) [52], integral field spectroscopy (IFS) [53], image-replicating imaging spectrometry (IRIS) [54], filter stack spectral decomposition (FSSD) [55], coded aperture snapshot spectral imaging (CASSI) [56], image mapping spectrometry (IMS) [57] and multi-spectral Sagnac interferometry (MSI) [58]. The only difference between these two categories is whether the filters are used. An example of CTIS system is shown in Fig. 2.7. The system consists of objective optics, collimator optics, a disperser and re-imaging lens. The objective optics images the sample at the field

stop. Then the collimator optics is used to collimate the light from the field stop and relay it to the disperser. The light is then imaged by the re-imaging lens to form the final image on a 2D detector array. It needs to be noted that, the data collected by the 2D detector array are not the exact spectral images. A 3D data cube in the form of commonly recognizable spectral images, with two spatial coordinates and one wavelength coordinate, needs to be reconstructed using an iterative method from a series of 2D projections that are created by the disperser.

Fig. 2. 8 Schematic of CTIS system. Figure adapted from reference [51]

Another example of CASSI is shown in Fig. 2.8. The sensing phenomena in CASSI is very simple. It usually consists of a coded aperture such as digital micromirror device (DMD), a dispersive element such as prism and a detector such as CCD. The coding is first applied to the objective data cube $f_0(x,y,\lambda)$ by coded aperture $T(x,y)$ and then modified by a dispersive element. The resulting dispersed data cube $f_2(x,y,\lambda)$ is integrated over the detector's spectral sensitivity to get the final compressive measurements. After acquiring the compressive measurements, the spatial-spectral data cube can be reconstructed from the compressive measurements by reconstruction algorithms, such as greedy pursuit [59], convex relaxation [60], Bayesian framework

[61, 62], nonconvex optimization [63] and brute force [64].

Fig. 2. 9 Schematic of CASSI system. Figure adapted from reference [65]

The primary disadvantage of snapshot spectroscopic imaging techniques in both categories is that data postprocessing is slow when the required spectral resolution is high. Another disadvantage is that snapshot techniques often require a very large detector array in order to properly sample a sufficient number of voxels in the datacube and those large detector arrays are usually expensive.

2.2.5 Narrow-band/Wide-band Measurements Followed by Spectral Reconstruction

Reconstruction of optical spectra from narrow-band/wide-band measurements is a potential way to realize spectral imaging with both high spatial and spectral resolution. Taking spectral reflectance imaging [66, 67] as an example for this method, a white light source is used to illuminate the sample and a camera is used to detect wide-band signals from the sample, in which the spectral information of the sample is suppressed to wide-band measurements. Then a reconstruction method is applied to the wide-band measurements to recover the spectral information. Various methods have been explored to reconstruct diffuse reflectance spectra from wide-band measurements, which includes finite-dimensional modeling[68], Wiener

estimation[66], etc. Among them, Wiener estimation is one of most frequently used methods because of its computational efficiency[69].

In general, there're two data sets involved in Wiener estimation, i.e. calibration data set and test data set. In the calibration data set, both narrow-band/wide-band measurements and the corresponding spectra for the same sample set are available; while there are only narrow-band/wide-band measurements in the test data set. The Wiener matrix needs to be extracted from the calibration data set first, which can be then applied to the narrow-band/wide-band measurements from the test data set to reconstruct the corresponding spectra. The method is much simpler compared with other snapshot spectral imaging techniques in computation while providing higher spectral resolution than multi-spectral imaging techniques. However, further work needs to be performed in the following aspects prior to broad applications. First, the accuracy of spectral information could be improved by exploiting information in the calibration dataset. Second, the measurement of the calibration data set is complex and costly, which needs to be simplified. Currently, a new calibration data set is often needed for every type of samples, which implies a huge burden and prevents this spectroscopic imaging approach from being widely adopted. Therefore, methods to avoid or reduce the measurements of calibration data set need to be investigated. If the above limitations can be overcome, the acquisition time can be significantly improved compared with traditional Raman imaging systems, as list in Table 2.1.

Table 2. 1 The comparison of time for point scanning, line scanning, wide-field imaging and our method

	Point scanning	Line scanning	Wide-field imaging	Our method
Acquisition time	11 h 24 min	12 min 34 s	46 min 22 s	1 min 26 s
Signal-to-noise ratio	29	77	14	80

Chapter 3: Modified Wiener Estimation for Spectral Reconstruction in Diffuse Reflectance Imaging of the Skin

[Copyright permission from: S. Chen, and Q. Liu, Modified Wiener estimation of diffuse reflectance spectra from RGB values by the synthesis of new colors for tissue measurements, J Biomed Opt 17(3), 030501 (2012).]

3.1 Introduction

As described in Section 2.1.2, the diffuse reflectance spectroscopy has been explored to noninvasively characterize tissue condition in recent years[70-73]. Diffuse reflectance spectra have been used to estimate the optical properties of tissues[74], which can be correlated with several important biophysical and biochemical parameters, such as hemoglobin concentration[75], tissue oxygenation[76] and average nuclei size[77], in tissues for diseases diagnosis[78, 79]. However, one significant disadvantage of traditional diffuse reflectance spectroscopy is slow data acquisition when diffuse reflectance spectra at multiple locations are required due to the employment of fiber-optic probes, which can only perform optical measurements point by point. Multi-spectral imaging has been used to acquire diffuse reflectance images at multiple wavelengths[80]. However, image acquisition can be still slow when the required spectral resolution is high. Although there have been efforts in the development of snap shot spectroscopic imaging techniques[81], the advantage of these techniques in quick image acquisition is usually compromised by the slow

postprocessing especially when the data dimension is large. Therefore, it is critical to develop a rapid spectral imaging technique, which could offer real time acquisition of diffuse reflectance spectra for tissue measurements in multiple locations without sacrificing the spectral resolution.

The reconstruction of diffuse reflectance spectra from color values taken by a color camera is a potential solution to this problem. In this approach, the color images of a large tissue area under white light illumination are captured in real time by a color camera, which typically includes the color values in Red, Green and Blue bands at each pixel. Each color band covers a relatively wide range of wavelengths thus the signal is typically strong for fast imaging. Several methods, e.g. finite-dimensional modeling[82], Wiener estimation[83] etc., have been explored to recover diffuse reflectance spectra from color values in multiple bands. Among them, Wiener estimation is one of most frequently used methods because of its time efficiency[84]. However, one serious problem of using this estimation method is that more than three color bands are usually necessary for sufficient accuracy[83]. Wiener estimation with only RGB bands is considered inadequate because of the underdetermined nature of the problem, in which three values corresponding to RGB colors are mapped to diffuse reflectance intensities at tens of or a few hundred wavelengths in a spectrum. As a result of inadequate Wiener estimation, the error in the estimated diffuse reflectance spectra could propagate and lead to larger errors in estimated optical properties[85], and subsequently in estimated tissue parameters for diagnosis. Therefore, a fast method for the accurate estimation of diffuse reflectance spectra is

needed in biomedical applications. In this letter, we propose a modified Wiener estimation method to address this problem by synthesizing new colors from the system matrix based on a calibration data set, which will provide additional information required for accurate Wiener estimation. The method has demonstrated significant improvement over traditional Wiener estimation in the estimation of diffuse reflectance spectra from color measurements acquired *in vivo* from human skin.

3.2 Materials and Methods

3.2.1 Traditional Wiener Estimation

Assume the diffuse reflectance spectrum can be written as s and s is an $n \times 1$ matrix, in which n is the number of wavelength in each spectrum. The transmittance spectra of the RGB filter set are represented by F , in which F is an $3 \times n$ matrix. The noise in color measurements is denoted by e that is an 3×1 matrix. Then the corresponding color measurements of the spectrum S generated by applying color filter set F is denoted by c , which can be expressed as

$$c = Fs + e \quad (3.1)$$

where c is an 3×1 matrix. In Wiener estimation, a Wiener matrix W ($n \times m$ matrix) is used to transform color measurements c ($m \times 1$ matrix) into the corresponding diffuse reflectance spectrum $\hat{s}$ ($n \times 1$ matrix),

$$\hat{s} = Wc \quad (3.2)$$

so that the mean square error between the original and estimated spectra is minimized.

The Wiener matrix $\mathbf{W}$ is given [86] by Eq. (6.5).

$$\mathbf{W} = \mathbf{K}_s \mathbf{F}^T (\mathbf{F} \mathbf{K}_s \mathbf{F}^T + \mathbf{K}_e)^{-1} \quad (3.3)$$

where

$$\mathbf{K}_s = \mathbf{E}[\mathbf{s}\mathbf{s}^T] \text{ , } \mathbf{K}_e = \mathbf{E}[\mathbf{e}\mathbf{e}^T] \quad (3.4)$$

In Eq. (3.3) and (3.4), the superscript “T” represents matrix transpose, the superscript “−1” represents matrix inverse and $E[\cdot]$ represents an ensemble average. Plugging Eq. (3.4) into Eq. (3.3) and ignoring the noise term yields

$$\mathbf{W} = \mathbf{E}[\mathbf{s}\mathbf{c}^T][\mathbf{E}[\mathbf{c}\mathbf{c}^T]]^{-1} \quad (3.5)$$

Once Wiener matrix W is created in the calibration stage, it can be applied to color measurements in the test stage to reconstruct the corresponding diffuse reflectance spectrum using Eq. (3.2).

3.2.2 Modified Wiener Estimation

Fig. 3. 1 Schematic of the calibration stage

This method consists of two stages and one postprocessing step, i.e. the calibration stage, the test stage and the selection step. In the calibration stage as shown in Fig. 3.1, we first calculate the system matrix and initial Wiener matrix using the calibration data which contain both measured RGB color values and the corresponding measured

diffuse reflectance spectra. The estimation of the system matrix is an over-determined problem thus should be accurate because a diffuse reflectance spectrum contains much more data points than RGB values. Diffuse reflectance spectra can be estimated from measured RGB color values by using the initial Wiener matrix. Then, by applying a synthetic absorption filter, which contains three absorption bands different from RGB filters, to the system matrix, a modified system matrix can be created, which is used to generate two separate sets of three new color values from the measured and estimated diffuse reflectance spectra. The transmission spectra of the three synthetic absorption filters were determined by Gaussian functions with different center wavelengths, i.e. 500 nm, 550 nm and 600 nm, and a standard deviation of 100 nm. The center wavelengths were selected to deviate from those of RGB filters in order to cover different information. Furthermore, the transmission spectra of these synthetic filters are significantly different from those of common RGB filters in a commercial color camera. Because of the high accuracy of the system matrix, the new color values estimated from the measured diffuse reflectance spectrum should be accurate thus could be seen as the reference new color values. The new color values generated from the estimated diffuse reflectance spectra are less accurate, which will be called estimated new color values to facilitate the discussion next. The relation between the estimated new color values and the reference new color values can be found by using the following two strategies: (A) to model each reference new color value as a second-order polynomial function of estimated new color values; (B) to record the differences in each color value between two sets.

Moreover, the reference new color values combined with the original RGB values are used to create a modified Wiener matrix for the second-round estimation of diffuse reflectance spectra in the test stage, which will be more accurate than using the RGB values alone because of the increase in the number of available color bands.

Fig. 3. 2 Schematic of the test stage

In the test stage as shown in Fig. 3.2, the initial estimated diffuse reflectance spectrum will be computed by applying the initial Wiener matrix to measured RGB values first, which is usually not sufficiently accurate because the initial Wiener matrix is based on RGB values alone. Estimated new color values will be generated from the initial estimated diffuse reflectance spectrum by applying the modified system matrix obtained in the calibration stage. Then the estimated new color values are corrected by the relation between the estimated new colors and the reference new colors found in the calibration stage to yield more accurate new color values as explained in the next paragraph, which is also called color correction. Then the corrected new color values jointly with the measured RGB values can be used to estimate more accurate diffuse reflectance spectra by applying the modified Wiener matrix obtained in the calibration stage.

The two strategies for finding the relation between the estimated new colors and the reference new colors are used as follows. To use the relation of the second order polynomial function, i.e. Strategy (A), estimated new color values are substituted into the second order polynomial functions obtained in the calibration stage to find new color values. To use the relation of recorded differences, i.e. Strategy (B), the difference values used to modify the estimated new colors are calculated by using the following equation:

$$D = \sum_{i=1}^n w_i d_i \quad (3.6)$$

where D denotes the difference values used to modify the estimated new colors, w_i and d_i denote the i -th weight and difference values for each point in the calibration data set, respectively. The weight w_i is calculated by the equation as follows:

$$w_i = \frac{l_i^{-1}}{\sum_{i=1}^n l_i^{-1}} \quad (3.7)$$

where l_i denotes the distance in the RGB color space between the measured color in the test data and the i -th color in the calibration data. From Eq. (3.6) and (3.7), the summation of w_i is 1, and the contribution of d_i to D is proportional to the similarity between the measured color in the test data set and the i -th color in the calibration data set.

Because the above two new color correction strategies used to correct estimated new color values generate different diffuse reflectance spectra, a simple selection step could be employed to find the more accurate one. In this selection step, the estimated spectra based on the above two strategies are multiplied by the original system matrix

to find the estimated RGB color values. The diffuse reflectance spectrum that yields color values closer to the measured ones will be selected as the final result.

3.2.3 System Information

Fig. 3. 3 Diagram of the system for taking color images and diffuse reflectance spectra. The following acronyms are used. SM: spectrometer; LS: light source; P: probe; S: sample.

This method was evaluated in the data measured *in vivo* from ten volunteers. All the data were measured by the system shown in Fig 3.3, which consists of a light source (HL-2000-FHSA, Ocean Optics, US), a spectrometer (USB4000, Ocean Optics, US), a fiber-optic probe (custom probe VIS/NIR, Ocean Optics, US) and a microscope (Eclipse TS100, Nikon, Japan) coupled with a color CCD camera (DS-Fi1, Nikon, Japan) in which the Bayer color filter array is used for RGB image acquisition. The probe was composed of one source fiber with a core diameter of 600 μm surrounded by ten detector fibers with a core diameter of 100 μm with a center-to-center separation of 413 μm . The measured diffuse reflectance spectrum was normalized to remove the wavelength dependence of system response by dividing the spectrum measured on the skin by the spectrum measured on a reflectance standard

(SRS-99-010, Labsphere, US). Then the data points in the spectrum were binned so that the spectral resolution was 5 nm from 400 nm to 700 nm. By using this system, both RGB color values and diffuse reflectance spectrum were measured from the same position on the skin to prevent the mismatch in measurements sites between two sets of measurements.

Fig. 3. 4 Typical skin images acquired by using the proposed system

A total of 200 skin sites from 10 volunteers, which includes 6 Chinese, 3 Indians, and 1 Caucasian, were measured in this experiment. Fig. 3.4 shows the typical skin images, i.e. light, medium and dark skin, acquired by using the proposed system. A leave-one-out cross validation strategy[87] was used to analyze the data to evaluate the modified Wiener estimation method in an unbiased manner. In this strategy, the measurements from one skin site were retained as the test data each time and other measurements from the rest of 199 skin sites were used as the calibration data set. This procedure was repeated until the measurements from every skin site have been tested once. The reason of using human skin measurements for calibration instead of color chart as in several other previous studies is that such calibration data similar to

the test data should yield better accuracy in estimated results[88]. The proposed method was coded and run in Matlab (R2008a, MathWorks, US).

3.3 Results and Discussions

A total of four methods, which includes the traditional Wiener estimation and the modified Wiener estimation methods using three different strategies for correcting estimated RGB values, were utilized to estimate diffuse reflectance spectra from given RGB values and their performance was compared against each other. The mean Root Mean Square Errors, i.e. RMSE, of estimated diffuse reflectance spectra relative to measured diffuse reflectance spectra for four methods are shown in Table 3.1. The representative spectra for the modified Wiener estimation method with the best and worst accuracy are shown in Fig. 3.5.

Fig. 3. 5 Comparison between the measured diffuse reflectance spectrum and the spectrum

Table 3. 1 Comparison of mean RMSE between the traditional Wiener estimation and modified Wiener estimation^a

	Traditional WE	Modified WE with Strategy A	Modified WE with Strategy B	Modified WE with the selection step
RMSE	1.11×10^{-2}	1.03×10^{-2}	1.06×10^{-2}	9.40×10^{-3}

^aWE is the acronym of Wiener Estimation

Table 3.1 shows that the modified Wiener estimation method reduces the RMSE by 7.2% or 4.5%, respectively, compared to the traditional Wiener estimation, when Strategies A or B is used to correct new RGB values. Moreover, a much larger reduction in the RMSE, i.e. 15.3%, is seen when the selection step is added. This infers that more accurate new color values yielded by the selection step are critical to the accurate estimation of diffuse reflectance spectra. It took 19.3 milliseconds to estimate the diffuse reflectance spectra from 400 nm to 700 nm with a resolution of 5 nm at 50 skin sites in a PC with Intel Core 2 CPU 2.4GHz, 2G RAM and Windows Vista operating system. This short estimation time implies the potential of performing real time estimation of diffuse reflectance spectra based on color images acquired from a large tissue area.

2.4 Conclusions

In conclusion, we have developed a modified Wiener estimation method by synthesizing new colors from the system matrix based on a calibration data set. The proposed method significantly improved the accuracy of estimated diffuse reflectance

spectra for RGB color values acquired from human skin. This method is fast thus it may allow real time estimation of diffuse reflectance spectra from a large tissue area when combined with color imaging, which provides a cost effective alternative to spectral imaging with the additional advantage of high spectral resolution.

Chapter 4: Sequential Weighted Wiener Estimation for Tissue Parameter Estimation

[Copyright permission from: S. Chen, X. Lin, C. Zhu and Q. Liu, Sequential weighted Wiener estimation for extraction of key tissue parameters in color imaging: a phantom study, J Biomed Opt 19(12), 127001 (2014).]

4.1 Introduction

In the previous Chapter, we have accurately reconstructed the diffuse reflectance spectra from the RGB values, which the key tissue parameters could be derived from the reconstructed diffuse reflectance spectra by several published methods. Therefore, we mainly investigated the feasibility to extract the key tissue parameters from the RGB values and their ratios directly, which the acquisition speed can be further improved.

Key tissue parameters, e.g. total hemoglobin concentration and tissue oxygenation, are important biomarkers in clinical diagnosis for various diseases[89]. The previous studies have shown that diffuse reflectance spectroscopy can be used to accurately recover these parameters noninvasively using different methods, such as Monte Carlo based inverse model[90], diffusion theory based model[91] and the lookup table method[13]. However, the common disadvantage of traditional diffuse reflectance spectroscopy is slow data acquisition when multiple locations need to be measured[92]. This is attributed to the employment of fiber-optic probes in traditional diffuse reflectance spectroscopy, which can only perform point measurements. Slow data acquisition prohibits the use of diffuse reflectance spectroscopy in those

applications in which the observation of fast changing phenomena is required.

Several spectral imaging techniques are developed to speed up the data acquisition. Multispectral imaging[36, 93, 94] captures image data at certain wavelengths using specific filters or instruments that are sensitive to particular wavelengths instead of the entire spectrum. The advantage of this technique is that both the spectral and spatial information can be recorded simultaneously[95]. However, the data acquisition is time consuming when high spectral resolution is required. This limitation originates from the employment of a wavelength selection/dispersion device in most imaging setups, for which the information at only one or a couple of wavelengths can be collected in one measurement. Snapshot hyperspectral imaging is a technique in which both the spatial and spectral information are acquired simultaneously in a snapshot[46] but in a coded manner. The key idea in snapshot hyperspectral imaging is that hyperspectral images can be captured all at once on a two-dimensional detector array. The disadvantage of snapshot hyperspectral imaging techniques is that data postprocessing is slow when the required spectral resolution is high. In addition, a large detector array is needed to properly sample a sufficient number of voxels and those large detector arrays are usually expensive. To overcome this limitation, spectral imaging can be realized by spectral reconstruction from wide-band measurements such as color imaging measurements, in which a method capable of fast and accurate spectral reconstruction is critical. Various methods have been explored to reconstruct diffuse reflectance spectra from wide-band measurements, which includes finite-dimensional modeling[68] and Wiener estimation (WE)[66].

Our previous study[92] has shown that diffuse reflectance spectra can be accurately recovered from in vivo color measurements on human skin. Therefore, it may be possible to directly derive tissue parameters from wideband measurements.

Multiple early studies[96, 97] have shown that hemoglobin concentration and oxygenation in tissue phantoms with known scattering properties can be estimated from color measurements in R(Red), G(Green), B(Blue) channels using a method involving Monte Carlo modeling and multiple steps of regression analysis. However, the scattering properties of tissues carry important physiological information[98], which are typically unknown and can vary significantly from one subject to another. To our best knowledge, there's no previous study in which both absorption and scattering properties are directly estimated from wide-band measurements in a tissue model with varying or unknown scattering properties.

In this study, we reported a method of sequential weighted Wiener estimation to directly extract total hemoglobin concentration (C_{tHb}), hemoglobin oxygenation (StO_2), scatterer density (α) and scattering power (β) from wide-band measurements. This method takes advantage of the fact that each parameter is sensitive to the color measurements to a different degree and attempts to maximize the contribution of those color measurements that are more likely to generate correct results in the process of Wiener estimation (WE). The method was evaluated on tissue phantoms with varying C_{tHb} , StO_2 and scattering properties to mimic human skin, in which a 3-CCD camera was used to capture the wide-band color measurements from the tissue phantoms. The results demonstrate excellent agreement between the estimated tissue

parameters and the corresponding reference values. Compared with the traditional Wiener estimation, the method of sequential weighted Wiener estimation shows significant improvement, especially for hemoglobin oxygenation estimation. This method possesses great potential in the noninvasive and real-time monitoring of tissue parameters in an imaging setup to investigate fast changing phenomena.

4.2 Materials and Methods

In this study, tissue phantoms with varying absorption, scattering and oxygenation were used to mimic the human skin. Both traditional WE and sequential weighted WE were tested to extract tissue parameters, including the total hemoglobin concentration (C_{tHb}), hemoglobin oxygenation (StO_2), scatterer density (α) and scattering power (β). The leave-one-out method was used for cross validation and the genetic algorithm was utilized to find the optimal combination of coefficients. All the post-processing steps, including the traditional WE, sequential weighted WE, leave-one-out method and genetic algorithm, were coded and run in Matlab (R2011b, MathWorks, Natick, Massachusetts, US). The details in each step are described as follows.

4.2.1 System Information

Fig. 4. 1 Schematic of the color imaging system with devices measuring pH and dissolved oxygen in the sample. The red lines with arrows represent light flow.

In this study, all the data were measured by the system shown in Fig. 4.1, which consisted of a white light source (UHP-Mic-LED-White, Prizmatix, Givat-Shmuel, IL), a 3-CCD (AT-200 GE, JAI, San Jose, California, US), a magnetic stirrer (97042-596, VWR, Radnor, Pennsylvania, US) and two linear polarizers (LPVISE100-A, Thorlabs, Newton, New Jersey, US). White light passed through the first linear polarizer, which was aligned such that its direction of polarization was parallel to the source-sample-detector plane. The illumination light was delivered at a fixed angle of 45 degrees onto a sample to avoid the collection of specular reflectance[99]. The second linear polarizer was put in front of the 3CCD in order to acquire signal with two different polarization directions (parallel or perpendicular to the incident polarization). The signal detected with parallel polarization contained contributions from both the superficial and deep regions inside the phantom, in which

photons responsible for contribution from the superficial are mostly scattered only single or a few times[100, 101]. Therefore, the signal detected with parallel polarization was expected to be sensitive to the scattering properties of the phantoms thus in turn to the scatterer density (α) and scattering power (β). In contrast, the signal detected with perpendicular polarization contained only light from deep regions inside the phantom, which was expected to be sensitive to the absorption properties of the phantoms due to the long optical path length and in turn to total hemoglobin concentration (C_{thb}) and hemoglobin oxygenation (StO_2). The sample was viewed by the camera right above, along the normal axis of the sample surface. A computer was connected to the camera to acquire the images of the sample, which contained the wide-band color measurements. Among these tissue parameters, hemoglobin concentration and oxygen saturation together determine the absorption coefficient spectrum according to Beer's law while the scattering amplitude and scattering power together determine the reduced scattering coefficient μ_s' for the whole visible spectrum (λ) by the following empirical equation[102, 103].

$$\mu_s'(\lambda) = \alpha \lambda^{-\beta} \quad (4.1)$$

The spectra of absorption and scattering coefficients together with the configuration of optical measurements determine the diffuse reflectance spectrum. After passing through the RGB (i.e. Red, Green and Blue) filters, the diffuse reflectance spectrum is transformed to RGB values, as shown in Eq. (4.2).

$$C = FS \quad (4.2)$$

where C are the RGB responses, F represents the transmission spectra of RGB filters

and S is the diffuse reflectance spectrum. Therefore, the RGB responses and their ratios are determined by these tissue parameters therefore contain the information of all the tissue parameters. A pH meter and thermometer (HI 9024, Sigma-Aldrich, St. Louis, Missouri, US) and a dissolved oxygen meter (HI 9142, Sigma-Aldrich, St. Louis, Missouri, US) were used to measure the pH value, temperature and oxygen level in the phantom sample, respectively, which were used to derive hemoglobin oxygenation. The relative emission spectrum of the white light source, the relative spectral response of the 3-CCD and the transmittance of the polarizers are shown in Fig. 4.2.

Fig. 4. 2 (a) Relative emission spectrum of white light source, (b) relative spectral response of the 3-CCD and (c) transmittance of the polarizer

4.2.2 Phantom Preparation

The optical properties of tissue phantoms were selected to mimic human skin, in which hemoglobin served as the absorber and polystyrene spheres served as scatterers.

Totally 162 liquid phantoms with 3 different hemoglobin concentrations, polystyrene spheres with 2 different scatterer sizes and 3 different volume concentrations, and 9

different hemoglobin oxygenations were measured. Three concentrations of hemoglobin (H0267, Sigma-Aldrich, St. Louis, Missouri, US) were 3.9 μM , 19.4 μM , 38.8 μM , which corresponds to absorption coefficients of 2.3 cm^{-1} , 11.5 cm^{-1} and 23 cm^{-1} at 413 nm. The volume concentrations of polystyrene spheres with 1- μm diameter (07310-1, Polysciences, Warrington, Pennsylvania, US) were adjusted to achieve the scattering coefficients of 54.8 cm^{-1} , 109.6 cm^{-1} and 253.5 cm^{-1} at 550 nm. The volume concentrations of polystyrene spheres with 0.5- μm diameter (07307-15, Polysciences, Warrington, Pennsylvania, US) were adjusted to achieve the scattering coefficients of 55.0 cm^{-1} , 110.0 cm^{-1} and 256.6 cm^{-1} at 550 nm. The optical properties of the skin phantoms used in this study were extracted from *in vivo* skin studies[104-106] and *in vitro* skin studies[107-109], in which the subjects were mostly Caucasians. Phosphate buffer with a *pH* of 6.0 was used as the solvent in the phantoms to keep a constant *pH* value, which maintained the relation between measured oxygen tension and hemoglobin oxygenation approximately unchanged when the temperature was fixed[89]. Hemoglobin oxygenation was varied by adding 5 mg/ml yeast (Dry Baker's Yeast, saf-instant, Marcq-en-Baroeul, Cedex, FR) to remove oxygen from hemoglobin. The recorded hemoglobin oxygenation values ranged from 10% to 90% with an interval of 10%. The phantoms were consistently stirred at a moderate speed on top of the magnetic stirrer during measurements to maintain a uniform distribution of oxygenation and scatterer distribution. The dissolved oxygen meter was used to monitor oxygen concentration in the phantoms. The measured oxygen concentration was converted to hemoglobin oxygenation by

applying Henry's law[110] and Kelman's equation[111, 112] with the required parameters, i.e. pH and temperature values measured by the pH meter and the thermometer. It was found that the pH and temperature of the phantoms were maintained at approximately 6.0 and 20°C, respectively, during the measurements.

4.2.3 Data Analysis

4.2.3.1 Sequential Weighted Wiener Estimation

In traditional WE, when ignoring the noise term, the Wiener matrix is formulated as

$$W = E[sc^T][E[cc^T]]^{-1} \quad (4.3)$$

where the superscript “T” represents matrix transpose, the superscript “-1” represents matrix inverse, $E[]$ represents the ensemble average, s represents the tissue parameters and c represents the wide-band measurements. More details for traditional WE have been reported elsewhere[113]. In the weighted Wiener estimation we developed, the ensemble average is replaced by the weighted average as shown in Eq. (4.4).

$$W = \sum_{i=1}^n w_i s_i c_i^T \sum_{j=1}^n (w_j c_j c_j^T)^{-1} \quad (4.4)$$

where w_i or w_j is the weights for the i -th or j -th set of calibration data. This weight is calculated according to the following equation:

$$w_i = \frac{D_i^m}{\sum_{i=1}^n D_i^m} \quad (4.5)$$

where D_i is the similarity between the tissue parameter value estimated from the test

data and the corresponding parameter value in the i -th set of tissue parameters in the calibration data and m is the power to adjust the contribution of D_i . The similarity D_i is calculated according to Eq. (4.6).

$$D_i = \frac{d_i^{-1}}{\sum_{i=1}^n d_i^{-1}} \quad (4.6)$$

where d_i denotes the difference between the tissue parameter value estimated from the test data and the corresponding parameter value in the i -th set of tissue parameters in the calibration data and m is the power to adjust the contribution of d_i . When multiple estimated tissue parameters need to be included in the weighting scheme, the corresponding similarity values will be calculated separately according to Eq. (4.6) and then averaged to yield a single similarity value. The accuracy of the estimation can be improved by using a large number of calibration data highly similar to the test data set. According to Eq. (4.5) and Eq. (4.6), any calibration data with a set of tissue parameter values very different from the test data would yield a tiny weight, which makes nearly negligible contribution to the weighted Wiener matrix by Eq. (4.4). According to the same equations, a larger m generates a more effective calibration data set, which are more similar to the test data set, than a smaller m does. However, if m becomes too large, the weight can be very small not only for those calibration data very different from the test data but also for those calibration data moderately similar to the test data by Eq. (4.5) and Eq. (4.6). As a result, the effective calibration data will be much less because only those very similar to the test data are retained. Such a small set of calibration data is likely to yield large errors in estimation when

the calibration data or the test data contain measurement uncertainty or noises. Therefore, the adjustment of m is the tradeoff between the population size of effective calibration data and the similarity between the calibration data set and the test data set, when the full calibration data set is fixed. The value of m was fixed at 5 in this study to achieve the tradeoff. According to Eq. (4.4) to Eq. (4.6), the summation of w_i is 1, and the contribution of a set of calibration data to the Wiener matrix is proportional to the similarity between the estimated tissue parameters in the test data set and the i -th set of tissue parameters in the calibration data.

Fig. 4. 3 Schematic of sequential weighted Wiener estimation

The schematic of the sequential weighted WE is shown in Fig. 4.3. This method consists of multiple steps of estimation that can be run iteratively, in which the parameters in the order of estimation are the scattering power (β), the total hemoglobin concentration (C_{tHb}), the scatterer density (α) and hemoglobin oxygenation (StO_2). The estimation error of each individual parameter when using traditional Wiener estimation decreases with this order in the calibration stage therefore this order will prevent the early estimation error from propagating to the later estimation. In the first step, the scattering power β is extracted using traditional WE method. Then, C_{tHb} is estimated using the weighted WE as in Eq. (4.4) and the

weights are calculated using Eq. (4.5) and Eq. (4.6) according to the difference between the β value estimated from the test data and the β values in the calibration data. After that, α is estimated using the weighted WE as in Eq. (4.4) and the weights are calculated using Eq. (4.5) and Eq. (4.6) according to the difference between C_{tHb} and β values estimated from the test data and these values in the calibration data. Finally, StO_2 is estimated using the weighted WE as in Eq. (4.4) and the weights are calculated according to the difference between C_{tHb} , α and β values estimated from the test data and these values in the calibration data. This completes one loop in the sequential weighted WE. If the estimated results are not satisfactory, the loop can be run again, in which β will be calculated again according to the weights calculated according to the similarity between C_{tHb} , StO_2 and α values estimated from the test data in the first loop and those values in the calibration data. All the steps can be repeated iteratively until the estimated results are acceptable.

4.2.3.2 Leave-one-out Method, Genetic Algorithm and Evaluation Criterion

The leave-one-out method[114] was used for cross validation. In this strategy, data measured from one tissue phantom is used for testing and data measured from other tissue phantoms are used for calibration. Then a new tissue phantom is selected for testing and the procedure is repeated until all the tissue phantoms have been tested.

Table 4. 1 Coefficients for estimation of tissue parameters¹

Indices	Coefficients
1 ~ 12	$R_{ }, G_{ }, B_{ }, R_{\perp}, G_{\perp}, B_{\perp}, R_{sum}, G_{sum}, B_{sum}, R_{sub}, G_{sub}, B_{sub}$
13 ~ 24	$R_{ }/(R_{ }+G_{ }+B_{ }), G_{ }/(R_{ }+G_{ }+B_{ }), B_{ }/(R_{ }+G_{ }+B_{ }), R_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}), G_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}), B_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}),$ $R_{sum}/(R_{sum}+G_{sum}+B_{sum}), G_{sum}/(R_{sum}+G_{sum}+B_{sum}), B_{sum}/(R_{sum}+G_{sum}+B_{sum}), R_{sub}/(R_{sub}+G_{sub}+B_{sub}), G_{sub}/(R_{sub}+G_{sub}+B_{sub}),$ $B_{sub}/(R_{sub}+G_{sub}+B_{sub})$
25 ~ 36	$R_{ }/G_{ }, R_{ }/B_{ }, G_{ }/R_{ }, G_{ }/B_{ }, B_{ }/R_{ }, B_{ }/G_{ }, R_{\perp}/G_{\perp}, R_{\perp}/B_{\perp}, G_{\perp}/R_{\perp}, G_{\perp}/B_{\perp}, B_{\perp}/R_{\perp}, B_{\perp}/G_{\perp}$
37 ~ 48	$R_{sum}/G_{sum}, R_{sum}/B_{sum}, G_{sum}/R_{sum}, G_{sum}/B_{sum}, B_{sum}/R_{sum}, B_{sum}/G_{sum}, R_{sub}/G_{sub}, R_{sub}/B_{sub}, G_{sub}/R_{sub}, G_{sub}/B_{sub}, B_{sub}/R_{sub},$ B_{sub}/G_{sub}

Because the ratios of wide-band measurements contained important information about hemoglobin[115, 116], we examined both the absolute values of wide-band measurements and their ratios. These two sets of data were named jointly as coefficients in Table 4.1. Genetic algorithm was used to find the optimal combination of the coefficients for the estimation of each tissue parameter. The optimization methodology proceeded in the following manner. First, a population of coefficient combination was initialized randomly. Second, traditional WE (for comparison) or sequential weighted WE was applied to extract the tissue parameters and the accuracy of the recovered tissue parameters was evaluated. The accuracy was quantified according to the relative root mean square error (RMSE) calculated from Eq. (4.7). Third, a new population of coefficient combination was generated according to the accuracy of the recovered tissue parameters, in which the coefficient combination yielding higher accuracy was more likely to become the parent of the new population. The crossover rate was 0.9 and the mutation rate was 0.1. The second and third steps were repeated iteratively until an optimized combination of coefficient was found.

¹ R: Red value; G: Green value; B: Blue value; ||: light with parallel polarization; $\perp$: light with perpendicular polarization; sum: || + $\perp$; sub:

$$\text{Relative RMSE} = \sqrt{\frac{\sum_{i=1}^n \left(\frac{i\text{-th estimated parameter} - i\text{-th reference parameter}}{i\text{-th reference parameter}} \right)^2}{n}} \quad (4.7)$$

4.3 Results

Fig. 4. 4 An example of raw image from liquid phantom (Absorption coefficient at 413 nm = 11.5 cm⁻¹, Scattering coefficient at 550 nm = 110.0 cm⁻¹, StO₂ = 90%)

Table 4. 2 Relative RMSEs in the parameters estimated using traditional WE and sequential weighted WE relative to the reference values

	Traditional WE	First-iteration weighted WE	Second- iteration weighted WE
Relative RMSE in C _{tHb}	0.1681	0.1193	0.0287
Relative RMSE in StO ₂	1.2712	0.2825	0.2726
Relative RMSE in α	0.2517	0.0501	0.0473
Relative RMSE in β	0.0151	0.0140	0.0060

Fig. 4.4 is an example of raw image acquired from liquid phantom. Table 4.2 shows the relative RMSEs in the parameters estimated using traditional WE and sequential weighted WE relative to the reference values. Out of four parameters estimated, i.e. C_{tHb}, StO₂, scatterer density α and scattering power β, the relative RMSEs increases from the lowest to the highest for traditional WE in the order of β, C_{tHb}, α and StO₂,

and the sequential weighted WE performs estimation in the same sequence as shown in Fig. 4.3. The first weighted WE denotes the sequential weighted WE with only one iteration and the second weighted WE denotes the sequential weighted WE with two iterations. The relative RMSEs in estimated tissue parameters after the first-iteration weighted WE are 71.0%, 22.2%, 19.9% and 92.7% of those after traditional WE for C_{thb} , StO_2 , α and β , respectively. The relative RMSEs after the second-iteration weighted WE are 24.1%, 96.5%, 94.4% and 49.3% of those after the first weighted WE for the same parameters. The decreases in the relative RMSEs of parameters indicate that the sequential weighted WE improves the estimation as the number of iteration increases.

Figure 4.5 shows graphically the comparison between estimated parameters and reference parameters in the traditional WE and sequential weighted WE. It is observed that the markers representing the weighted WE stay much more packed and closer to the ideal lines than those representing the traditional WE, which suggests that the weighted WE, especially after two iterations, is more effective in the quantification of these parameters.

Fig. 4. 5 (a) Total hemoglobin concentration C_{tHb} , (b) hemoglobin oxygenation StO_2 , (c) scatterer density α and (d) scattering power β estimated using the traditional WE and weighted WE as a function of the expected value. The legend “1st weighted” means the result after the first iteration of weighted Wiener estimation and the legend “2nd weighted” means the result after the second iteration of weighted Wiener estimation.

The best combination of coefficients for estimating C_{tHb} , StO_2 , α and β in the traditional WE, the first-iteration weighted WE and the second-iteration weighted WE are shown in Table 4.3. It is interesting to observe that not only the best combination but also the number of coefficients in the best combination change from the traditional WE to the weighted WE as well as from the first iteration to the second iteration in the weighted WE.

Table 4. 3 The best combination of coefficients for estimating C_{tHb} , StO_2 , α and β in

traditional WE, the first-iteration weighted WE and the second-iteration weighted WE

	Traditional WE	First-iteration weighted WE	Second-iteration weighted WE
C_{tHb}	$B_{ }, R_{\perp}, R_{sum}, R_{ }/(R_{ }+G_{ }+B_{ }),$ $R_{sum}/(R_{sum}+G_{sum}+B_{sum}), G_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $G_{sub}/(R_{sub}+G_{sub}+B_{sub}), B_{ }/G_{ },$ $G_{sum}/R_{sum}, R_{sub}/G_{sub}, B_{sub}/G_{sub}$	$R_{ }, B_{ }, R_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}), R_{\perp}/G_{\perp},$ $R_{\perp}/B_{\perp}, G_{\perp}/R_{\perp}, G_{\perp}/B_{\perp}, B_{\perp}/R_{\perp},$ $R_{sum}/B_{sum}, B_{sum}/G_{sum}$	$R_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}), G_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}),$ $R_{\perp}/G_{\perp}$
StO_2	$R_{ }, R_{\perp}, R_{sum}, B_{sum}, R_{sub}, B_{sub},$ $R_{ }/(R_{ }+G_{ }+B_{ }), B_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $G_{ }/R_{ }, G_{\perp}/B_{\perp}$	$R_{sum}, B_{sum}, R_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $G_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $B_{sum}/(R_{sum}+G_{sum}+B_{sum}), G_{sum}/R_{sum},$ B_{sum}/R_{sum}	$B_{sum}, R_{sum}/(R_{sum}+G_{sum}+B_{sum}), G_{\perp}/R_{\perp},$ G_{sum}/B_{sum}
α	$R_{\perp}, B_{sum}, G_{sub}, G_{ }/(R_{ }+G_{ }+B_{ }),$ $G_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}), B_{sub}/(R_{sub}+G_{sub}+B_{sub}),$ $R_{ }/G_{ }, R_{sum}/G_{sum}, G_{sum}/B_{sum}, R_{sub}/B_{sub},$ G_{sub}/B_{sub}	$R_{sum}, B_{sum}, R_{ }/(R_{ }+G_{ }+B_{ }),$ $B_{ }/(R_{ }+G_{ }+B_{ }), R_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}),$ $G_{sum}/(R_{sum}+G_{sum}+B_{sum}), R_{ }/G_{ }, B_{ }/G_{ },$ $B_{sum}/R_{sum}, B_{sum}/G_{sum}$	$B_{ }, R_{\perp}, G_{sum}, B_{sum}, R_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}),$ $G_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}),$ $R_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $G_{sub}/(R_{sub}+G_{sub}+B_{sub}), R_{\perp}/G_{\perp}, G_{\perp}/R_{\perp},$ $B_{sum}/R_{sum}, R_{sub}/G_{sub}$
β	$R_{sum}, R_{sub}, R_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}),$ $R_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $B_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $R_{sub}/(R_{sub}+G_{sub}+B_{sub}),$ $G_{sub}/(R_{sub}+G_{sub}+B_{sub}),$ $B_{sub}/(R_{sub}+G_{sub}+B_{sub}), G_{sub}/R_{sub}, G_{sub}/B_{sub},$ $B_{sub}/R_{sub}, B_{sub}/G_{sub}$	$R_{ }, G_{ }/(R_{ }+G_{ }+B_{ }),$ $G_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $B_{sub}/(R_{sub}+G_{sub}+B_{sub}), R_{ }/G_{ }, R_{\perp}/G_{\perp},$ $R_{sum}/G_{sum}, R_{sum}/B_{sum}, G_{sum}/R_{sum},$ $R_{sub}/G_{sub}, G_{sub}/B_{sub}$	$R_{ }, R_{ }/(R_{ }+G_{ }+B_{ }),$ $B_{ }/(R_{ }+G_{ }+B_{ }), B_{\perp}/(R_{\perp}+G_{\perp}+B_{\perp}),$ $B_{sum}/(R_{sum}+G_{sum}+B_{sum}),$ $G_{sub}/(R_{sub}+G_{sub}+B_{sub}), G_{ }/R_{ }, G_{ }/B_{ },$ $R_{sub}/G_{sub}, B_{sub}/R_{sub}, B_{sub}/G_{sub}$

4.4 Discussions

According to Table 4.2 and Fig. 4.5, all the estimated parameters show significant improvement when using the sequential weighted WE. The traditional WE fails to recover the StO_2 , whereas the sequential weighted WE shows good performance for the recovery of StO_2 . The improvement can be attributed to the optimal selection of the calibration data set, in which a more appropriate Wiener matrix is created compared with the traditional WE. In addition, with the increasing number of iterations in the sequential weighted WE, all the estimated parameters show improved accuracies. This is due to the better choices of weights used in Eq. (4.4) to Eq. (4.6), i.e. more contribution from the calibration data with a higher similarity to the test data, which means a more appropriate Wiener matrix can be created. However, more iterations require more computation time, thus a trade-off is needed between the estimation accuracy and the time cost in a clinical application.

In all the data shown up to this point, the similarity values calculated separately for each parameter according to Eq. (4.6) were averaged to represent the similarity of the multiple parameters in each set of data. We also evaluated the possibility of use the multiplication of individual similarity values instead of the averaging of them to generate a single similarity indicator for the multiple parameters in each set of data as shown in Table 4.4, in which multiplication was found to yield faster convergence than averaging. The results in Table 4.4 show significant improvement on the estimation of C_{tHb} and β , slightly improvement in the estimation of α , and slight degradation in StO_2 estimation accuracy.

Table 4. 4 Relative RMSEs in the parameters estimated using sequential weighted WE based on averaging and multiplication for the similarity indicator of multiple parameters

	First-iteration weighted WE (averaging)	Second- iteration weighted WE (averaging)	First-iteration weighted WE (multiplication)	Second- iteration weighted WE (multiplication)
Relative RMSE in C_{tHb}	0.1193	0.0287	0.1193	0.0151
Relative RMSE in StO_2	0.2825	0.2726	0.2978	0.3070
Relative RMSE in α	0.0501	0.0473	0.0430	0.0421
Relative RMSE in β	0.0140	0.0060	0.0065	0.0030

According to Table 4.3, all the combination of parameters contains ratio values of wide-band measurements, which confirms the importance of ratio values for the recovery of all tissue parameters. However, there's no obvious preference in the polarization state of light and/or specific ratio values for the recovery of any individual tissue parameter. The possible reason is that although certain coefficient is more important for a given tissue parameter, information from other coefficients is still needed for accurate estimation in this particular imaging setup. For example, the

scattering properties including α and β are generally considered to be more sensitive to the measurements in parallel polarization that involves most contribution from the shallow sample volume. In principle, the measurements in perpendicular polarization should not appear in the optimal combination of coefficients for estimating the scattering properties. However, the small detector, which is determined by the pixel size of the camera in this imaging setup, could break this principle since a small detector also preferentially detects signals from the shallow sample volume. As a result, multiple coefficients involving the measurements of perpendicular polarization are also included in the optimal combination for estimating the scattering properties, α and β , in Table 4.3. Interestingly, only the measurements of perpendicular polarization or the sum of measurements in both polarizations show up in the optimal combination for estimating C_{tHb} and StO_2 . The excellent performance of the sequential weighted Wiener estimation can be attributed to two factors. One factor is that the proposed method based on Wiener estimation takes the advantage of prior information about samples contained in the Wiener matrix[117]. The Wiener matrix is created in the calibration stage, in which the tissue parameters measured from similar samples are used and associated with color measurements. The other factor is that the sequentially introduced weights yield an optimal calibration data set for each sample, thus a more appropriate Wiener matrix is created. This is also the advantage compared with the previous approach for the same problem[97], in which fixed scattering coefficients and anisotropy factors were used. Because the color measurements are influenced jointly by all tissue parameters, the estimation of one

particular tissue parameter, especially StO_2 , which is essentially done by comparison between the test data and the similar data in the calibration data set, can be easily hindered by small perturbation or error in other tissue parameters. In this situation, the calibration data set created by the weighting step in the sequential weighted WE, which is more similar to the test data than the raw calibration data set, will yield a Wiener matrix that is more robust to perturbation in the color measurements or other tissue parameters. Moreover, it is adaptive to different test data. Compared with the sequential weighted Wiener estimation, the traditional Wiener estimation only need to create the Wiener matrix once. Therefore, the computing time for the sequential weighted Wiener estimation is around 50 times of that for the traditional Wiener estimation in this study. However, this problem could be solved by the prior calculation of the weighted Wiener matrix, which can be stored in a database according to the corresponding weights. When certain weights appear, the Wiener matrix can be directly retrieved from the database, then the computing time can be reduced dramatically.

Although the use of the calibration data set is a potential limitation of this method, it should be pointed out that the calibration data set is often available in most biomedical applications. This fact has been utilized in many earlier researches in medical diagnostics[118, 119]. However, when a totally new system (with a new light source and/or a new color CCD) is used, the instrument to instrument difference can degrade the estimation accuracy, since the calibration data set and the test data set were measured by using different systems. The problem can be solved by converting

the RGB values into the CIE (International Commission on Illumination) XYZ color space. The CIE XYZ color space is a device-independent color space[120]. The RGB values are transformed to CIE XYZ values by a transform matrix T as shown in Eq. (4.8).

$$\begin{bmatrix} X \\ Y \\ Z \end{bmatrix} = T \begin{bmatrix} R \\ G \\ B \end{bmatrix} \quad (4.8)$$

The transformation matrix T is dependent on the imaging system, which can be determined according to the measurements of a standard color chart using the imaging system. The standard color chart is supplied commercially with the corresponding CIE XYZ values. When the imaging system is changed, one just needs to obtain a new transformation matrix T by measuring the standard color chart again. The new transformation matrix will be then used to convert all color measurements from other samples to the CIE XYZ values, which is device independent thus would be consistent regardless of the imaging system. This method will solve the problem of switching the imaging system from one to another. The detailed information of this method can be found in a published paper[96]. For color reading variation due to factors other than the system, for example, the slightly variation in the imaging distance or angle, a single standard measurement can be applied to simplify the calibration step. The standard measurements are the measurements of a diffuse reflectance standard by using the current system and the new system. And the color response can be corrected using Eq. (4.9).

$$C' = C_{new} \frac{S_{cur}}{S_{new}} \quad (4.9)$$

where C' is the corrected color measurements, C_{new} is the color measurements from the new system, S_{cur} and S_{new} are the standard measurements using the current system and new system respectively. After the correction, the calibration data set acquired by the current system can be still used and the original Wiener matrix would remain effective. Another estimation degradation factor from ambient light could be reduced by subtracting the background measurement from any sample measurement. For every sample measurement, a subsequent background measurement was taken immediately then subtracted from the sample measurement, which has been shown effective in reducing the influence of ambient light.

Compared with diffuse reflectance spectroscopy based methods, the results for traditional Wiener estimation do look worse. However, the sequential weighted Wiener estimation improved the relative RMSEs over traditional Wiener estimation by an average of 4.6 fold, ranging from 0.6% to 27%. The relative RMSEs generated by the sequential weighted WE are comparable to the typical performance of diffuse reflectance spectroscopy based methods[13, 90, 121, 122], in which the relative RMSEs were approximately ranged from 1% to 15%. Although optical spectroscopy for tissue characterization in a point measurement setup has been investigated intensively to derive key tissue parameters for a couple of decades, color imaging for the same purpose has not been fully explored, which is evident from a limited number of relevant publications. Compared to the point measurement setup, we anticipate that our method will be advantageous in the speed of data acquisition when employed for extraction of tissue parameters in a large field of view and high spatial resolution. In

addition, the color imaging setup acquires images much faster than those more expensive spectral imaging setups. Our method requires only one color image, based on which tissue parameters at each pixel can be reconstructed. Therefore it may allow non-invasive and real-time monitoring of tissue parameters in a large tissue area to investigate fast changing phenomena.

The proposed method can be used in the clinical setting for various applications such as the assessment of skin viability. This study is a pilot study to verify the feasibility of tissue parameter extraction from RGB responses and their ratios and the efficiency of the proposed estimation algorithm. Therefore, a simplified phantom model was selected. Based on the feasibility of this study, the calibration data may need to be obtained from a more realistic tissue phantom model in clinical applications. For example, a series of two-layered skin phantoms including melanin in the epidermis with a range of epidermal thicknesses and optical properties in both layers will yield a better set of calibration data for clinical measurements. Since such a multi-layered phantom will contain much more parameters, a different optical system such as the depth sensitive multifocal color imaging system developed by our group[123] and a sequential estimation method[124] will be needed to selectively acquire color images from each layer to improve the accuracy of each estimated parameter. Although the additional melanin content could affect the color response and in turn the final estimation result, we believe that such an effect would be negligible if part of the calibration data set contains similar melanin content. According to our previous experiment, the average relative RMSE of C_{tHb} , α and StO_2 would increase from 7.7%

to 11.6% if the scattering power β was included as one free parameter instead of a known parameter. Therefore, it is expected that the average RMSE of other parameters may change in a similar trend with melanin concentration as an additional free tissue parameter. Considering the volume fraction range of melanin (1-3%) in the epidermis of the light skin[125] and the ratio (15%) between the epidermal thickness[126] and the typical penetration depth of our system, the total absorption coefficient of the skin may vary from 0.7 to 2.2 cm^{-1} at 550 nm due to the volume fraction variation in melanin. According to the semi-empirical model[127], the absorption coefficient variation can be converted to a decrease of around 20% in diffuse reflectance intensity at 550 nm and the same percent changes in R, G and B color values on average. Such a decrease in the R, G and B color values may yield an increase of about 3% in the relative RMSE according to the relationship between the effective signal to noise ratio and the relative RMSE we estimated (results not shown). Because the optical properties phantoms were extracted mainly from Caucasians, it is recommended to create a separate calibration dataset for each different skin type, for example, skin type I-VI[128]. In this manner, the influence of color changes caused by melanin in different skin types on the proposed method will be minimized.

Besides diffuse reflectance imaging, this method can be extended to fluorescence or Raman imaging to extract the concentrations of fluorophores or Raman scatterers. Fluorescence or Raman spectral measurements can be replaced by multiple wide-band or narrow-band measurements to enhance the signal to noise ratio and speed up data acquisition. This has been demonstrated previously for spontaneous Raman

spectra[113] in which the full Raman spectrum was reconstructed from multiple narrow-band measurements with small root mean square errors.

4.5 Conclusion

In this chapter, we developed a method of sequential weighted Wiener estimation to estimate key tissue parameters including total hemoglobin concentration (C_{tHb}), hemoglobin oxygenation (StO_2), scatterer density (α) and scattering power (β) from wide-band color measurements. Tissue phantoms with varying oxygenation were measured to evaluate this method. The estimated tissue parameters show excellent agreement with the reference values. Compared with the traditional Wiener estimation, the sequential weighted Wiener estimation shows significant improvement, especially for hemoglobin oxygenation. Moreover, the new method allows the estimation of the scattering properties, which overcomes the limitation of assuming known scattering properties or fixed scattering properties in the previous reports. This method is fast thus it may allow real time monitoring of key tissue parameters in a large tissue area when combined with color imaging. A future study will be conducted on a more complex multi-layered phantom with both melanin and hemoglobin as the absorbers.

Chapter 5: Spectral Diffuse Reflectance and Autofluorescence Imaging for Early Prediction of Flap Occlusion

5.1 Introduction

In Chapter 4, we have successfully extracted key tissue parameters from RGB values and their ratios, in which those key tissue parameters are important biomarkers in clinical diagnosis for various diseases. The result suggests that clinical diagnosis based on narrow-band measurements and their ratios may be feasible. In this study, we intend to investigate the feasibility of predicting flap occlusion early based on the narrow-band measurements and their ratios.

A skin flap is composed of skin and subcutaneous tissue that is transferred from one part of the body to another, with a connection to the body or vascular pedicle preserved for its blood supply [129]. The use of flap transfer has become a common and effective technique for reconstructing or replacing damaged tissues[130]. Flap transfer is often performed when a patient suffers tissue loss or damage due to burning, wound and deformities. The previous studies showed that 6% to 25% of flaps required surgical re-exploration for vascular compromise and approximately 10% of flaps were not salvageable [131, 132]. Vascular occlusion is the major reason for flap failure [133], which causes pain to patients and induces high medical cost. Early prediction of flap occlusion will allow a surgeon to take necessary precaution to reduce the rate of flap failure. Therefore, the early prediction of flap occlusion, which is the precondition of flap salvage, is critical to flap prognosis[134].

There're two types of ischemia: venous occlusion and arterial occlusion. According to the clinical observation, venous occlusion usually cause more severe damage to tissue viability than arterial occlusion. The current clinical method for the assessment of flap status is clinical examination by checking capillary refill, flap color, temperature, capillary turgidity etc. [135]. However, such examination is subjective because it relies heavily on the clinician's experience. What's more, it is difficult to monitor the flap condition using clinical examination continuously because clinical examination involves significant human labor. To address this problem, several instrumental methods have been developed for monitoring the flap condition. Laser Doppler imaging or similar techniques, as most commonly used complementary tools for tissue viability assessment, has been used to measure blood flow non-invasively[136, 137]. However, oxygen tension was shown more reliable as a diagnostic tool for interpretation of flap occlusion [138], which cannot be measured by laser Doppler imaging or similar techniques.

Diagnostic techniques based on optical spectroscopy have the potential to accurately evaluate tissue viability because these techniques are sensitive, noninvasive and quantitative[139]. Diffuse reflectance spectroscopy has been a widely used non-invasive technique in biomedical optics applications in recent years[140-143]. The previous study has shown that diffuse reflectance spectra can be used to provide information related to the optical properties of tissues [104], which can be correlated with several important biophysical and biochemical parameters, such as hemoglobin concentration[144], tissue oxygenation[145] and average nuclei size[146], in tissues

for diseases diagnosis[78, 79]. Autofluorescence spectroscopy is another well known spectroscopy technique for tissue assessment, which is based on detection of emitted fluorescence by endogenous fluorophores (tryptophan, tyrosine, phenylalanine, nicotinamide adenine dinucleotide, flavin adenine dinucleotide, collagen, elastin, and porphyrins) existing in tissues [18]. While NADH and FAD are indicators of cellular metabolism [147], collagen and elastin are two structural proteins [148]. Fluorescence spectra provide the quantitative information of these fluorophores and the corresponding fluorescence peaks are sensitive to metabolic and structural changes. Because diffuse reflectance and autofluorescence spectroscopy offer complementary information about tissue status, the combination of the two may be advantageous in terms of tissue assessment, which may yield more accurate tissue assessment.

While optical spectroscopy techniques have demonstrated many advantages, the common disadvantage of traditional optical spectroscopy is slow data acquisition when multiple locations need to be measured in a large tissue region. This is attributed to the employment of fiber-optic probes in traditional optical spectroscopy, which can only perform point measurements. It is time consuming when a large tissue region needs to be measured with high spatial resolution. In this case, a fast spectral imaging technique will be desired.

In response to the need stated above, the objective of this chapter is to develop a multispectral imaging system that combines autofluorescence and diffuse reflectance measurements for the early prediction of flap occlusion. Such a system will be helpful for assisting flap design and facilitating accurate timing of flap division.

5.2 Materials and Methods

5.2.1 System Information

Figure 5.1 shows the schematic of the experimental set-up of the combined diffuse reflectance and autofluorescence imaging system. The autofluorescence imaging module was comprised of collimation arrangement, filter wheel and electron multiplied charge coupled device (EMCCD). A 1-W 405nm laser was used as the excitation light source, which was passed through a 405-nm band pass filter with a bandwidth of 10 nm. The excitation light was then collimated using the combination of convex, plano-concave and plano-convex lens (L1) with 25.4 mm diameter each. After reflected by a dichroic mirror, the excitation light was passed through lenses L2 and L3 both with a focal length of 100 mm to maintain collimation. Then the light was reflected by a 70/30 beam splitter to illuminate the sample uniformly. The autofluorescence light of the sample was deflected by the beam splitter to transmit through the lenses L3, L2, dichroic mirror, 405-nm notch filter and filter wheel in the backward direction and imaged by the EMCCD placed behind the filter wheel. The 405-nm notch filter was used to eliminate the excitation light. The filter wheel contained 8 filters to cover the visible spectral region. Those filters include 1 emission filter (MF460-60) from Thorlabs (Newton, New Jersey, US), 1 fluorescence filter (84-097) from Edmund Optics (Barrington, NJ, US), 1 bandpass filter (NC282770) from Chroma Technique Cooperation (Bellows Falls, VT, US) and 5 bandpass filters (FF01-445/20-25, FF01-500/15-25, FF01-542/50-25, FF01-572/15-25,

FF01-580/14-25) from Semrock (Rochester, NY, US). The EMCCD attached with an imaging lens (Kreunach Xenon 0.95/25) captured an area of 22mm in diameter.

The diffuse reflectance imaging module was comprised of a white light source (UHP-Mic-LED-White, Prizmatix, Israel) and a 3CCD (AT-200 GE, JAI, USA). The illumination light was delivered at a fixed angle of 45 degree onto a sample to avoid the collection of specular reflectance. The 3CCD was placed on top of the beam splitter to collect the diffuse reflectance signal coming from the skin flap. The major components in this system, including the laser, white light source, EMCCD, 3CCD and filter wheel, were controlled by a customized Labview program.

Fig. 5. 1 Schematic of the diffuse reflectance and autofluorescence imaging system

5.2.2 Animal Preparation

Fourteen Sprague-Dawley rats were used in the animal study. The animal study followed the SingHealth Institutional Animal Care and Use Committee's requirements. All the procedures, including both surgery and measurements, were performed when the rats were placed under 1.2% to 2% isoflurane inhalational anesthesia. After induction of anesthesia, the animal was placed in a supine position. The abdominal and inguinal regions of the rat were shaved and depilated chemically. A 3 cm x 3 cm square cutaneous flap was marked in both groins using a surgical skin marker. Baseline optical measurements and images were obtained prior to surgery. The skin was prepared with 1% povidine iodine alcohol solution. Bilateral groin skin flaps were designed and raised based on blood supply from the epigastric vessels on each side. Tegaderm™ (3M) was placed between the wound bed and the flaps to prevent revascularization from the wound bed. Either the artery, or vein, or neither vessels (control) were ligated and transected according to the study group allocation. The flaps were then secured to their original positions and the wound edges closed with non-absorbable sutures. Baseline optical measurements were obtained again after flap elevation. Optical measurements were taken under anesthesia at the desired intervals. Final skin viability was determined clinically 24 hours after flap elevation, prior to euthanization of the animals. There're totally 28 flaps, which can be divided into three groups, i.e. the arterial occlusion, venous occlusion and control. One flap was excluded due to failure of the surgery. The number of arterial occlusion, venous occlusion and control flaps were 15, 8 and 4, respectively. Optical measurements

were performed once every 15 minutes in the first one hour, then once every 2 hours till the third hour and once every 3 hours until the twelfth hour. The baseline measurements were also acquired before the occlusion in each rat.

5.2.3 Data Analysis

All the color/narrow-band measurements in both diffuse reflectance imaging and autofluorescence imaging and their ratios were first divided by the corresponding baseline measurements to minimize the variation across animals. Then, principal component analysis (PCA) was utilized to extract the principal component (PC) scores to achieve data compression. The Wilcoxon rank sum test was performed to identify the principal component scores showing statistically significant differences between occlusion and control groups as well as between arterial occlusion and venous occlusion groups. The selection of the principal component scores was based on the following procedure. If there's no principal component score with a p-value less than 0.05 in the first several minutes or hours, the principal component score with the minimum p-value would be selected. If there're principal component scores with p-value less than 0.05 in the first several minutes or hours, all the principal component scores with p-value less than 0.05 would be selected. The selected principal component scores were used to group the data using a scheme of two-step binary classification. The two-step scheme first classified the flaps into the occlusion group and control group. Then, a second step was performed to classify those flaps in the occlusion group into the arterial occlusion group and venous occlusion group. The

two-step scheme can improve the overall classification accuracy by selecting optimal differentiators in each step.

5.3 Results and Discussions

Fig. 5. 2 Typical flaps with venous occlusion and arterial occlusion shown at 12 hours post flap elevation. The fluorescence image is acquired by using the filter (MF460-60).

Fig. 5.2 shows the typical venous occluded flap, arterial occluded flap and control flap at 12 hours post flap elevation. Table 5.1 and Table 5.2 show the principal component scores selected by Wilcoxon rank sum test in diffuse reflectance imaging and autofluorescence imaging, respectively. For classification between the occlusion and control groups, there are only a couple of principal component score selected from 15 minutes to 3 hours after occlusion. The finding can be explained as follows. The difference between the occlusion and control group is most significant

immediately after the occlusion. However, the recovery of flaps may reduce the distinction between the occlusion and control groups with time. In contrast, for the classification between the arterial occlusion and venous occlusion groups, principal component scores from both diffuse reflectance imaging and autofluorescence imaging showing significant differences can be found from 15 minutes to 3 hours after occlusion, due to different injury levels in the skin caused by venous occlusion and arterial occlusion.

Table 5. 1 Principal component scores selected by Wilcoxon rank sum test in diffuse reflectance imaging*

	5 min	15 min	30 min	45 min	1 h	3 h
Classification between occlusion and control	PC4	PC4	N. A.	N. A.	N. A.	N. A.
Classification between arterial and venous occlusion	PC3	PC2, PC3	PC2, PC3	PC2, PC3	PC2, PC3	PC2

*PC_i represents the i-th score calculated from PCA.

Table 5. 2 Principal component scores selected by Wilcoxon rank sum test in autofluorescence imaging*

	5 min	15 min	30 min	45 min	1 h	3 h
Classification between occlusion and control	PC3	PC8	N. A.	PC15	N. A.	N. A.
Classification between arterial and venous occlusion	PC4	PC3	PC3	PC2	PC2	PC2, PC5

*PC_i represents the i-th score calculated from PCA.

Table 5.3 shows the overall classification accuracy at different time points using only

diffuse reflectance imaging (R), only autofluorescence imaging (F) and both (R+F). When using only diffuse reflectance imaging data, the classification accuracy remains almost unchanged with time. When using only autofluorescence imaging data, the classification accuracy dramatically increased from 5 minutes to 15 minutes after occlusion, which then reaches a maximum of 77.8% at 45 minutes after occlusion. When using the combination of diffuse reflectance imaging and autofluorescence imaging data, the accuracy improves significantly compared with using only diffuse reflectance imaging or only autofluorescence imaging data and the accuracy reaches a maximum of 92.6% at 45 minutes after occlusion. The significant improvement can be explained as follows. The diffuse reflectance imaging contains the information about tissue oxygenation and total hemoglobin concentration as described in Chapter 4, while the autofluorescence imaging represents the information about tissue metabolic rate by measuring endogenous fluorophores including NADH and FAD. Therefore, diffuse reflectance imaging and autofluorescence imaging provide complementary information about the tissue status and the combination of those two techniques provides higher classification accuracy.

Table 5. 3 Overall classification accuracy at different time points after occlusion by using only diffuse reflectance imaging (R), only autofluorescence imaging (F) and

both (R+F)

Time Accuracy	5 min	15 min	30 min	45 min	1 h	3 h
R	66.7%	66.7%	66.7%	63.0%	66.7%	66.7%
F	55.6%	74.1%	70.4%	77.8%	77.8%	74.1%
R+F	66.7%	81.5%	85.2%	92.6%	85.2%	70.4%

5.4 Conclusions

In this chapter, we demonstrated the feasibility of using diffuse reflectance imaging combined with autofluorescence imaging to predict flap occlusion. The combination of those two imaging techniques shows significant advantage over using either one of them alone. Compared with the traditional spectroscopic techniques, the proposed imaging method is non-contact and offers a large field of view with high spatial resolution. Therefore, it may yield significant advantages when introduced into the clinical setting.

Chapter 6: Wiener Estimation for Spectral Reconstruction in Raman Imaging

[Copyright permission from: S. Chen, Y. H. Ong and Q. Liu, Fast reconstruction of Raman spectra from narrow-band measurements based on Wiener estimation, J Raman Spectrosc 44(6), 875-881 (2013).]

6.1 Introduction

In Chapter 3, we have successfully reconstructed the diffuse reflectance spectra from RGB values. In this chapter, we extend the proposed spectroscopic imaging technique to the Raman imaging.

Raman spectroscopy is a non-invasive spectroscopic technique, which provides quantitative information about molecular vibrations that can be used to investigate the chemical composition and structure of molecules within tissues and cells[149, 150]. Recently, with the development of the Raman spectroscopy in the field of biomedical optics, Raman spectroscopy has become a diagnostic or analytic tool for various diseases[28, 29], e.g. cancer[151, 152], bone diseases[153], and skin diseases[154]. However, the wide clinical employment of Raman based diagnostic techniques is hindered by the inherently weak Raman signals in biological samples, and consequently, slow data acquisition in Raman measurements[155]. Long time required for Raman measurements prohibits the use of Raman spectroscopic imaging for the investigation of fast changing phenomena in biological samples.

Several solutions have been explored to overcome this limitation. One way to speed

up Raman acquisition is to acquire Raman spectra from all the locations along a line simultaneously [156, 157]. In this method, a cylindrical lens is used to focus laser light onto or a laser spot is scanned along a line to create a line focus. The Raman light generated in the line focus will be dispersed by a grating onto a CCD. While one dimension of the CCD covers all the wavenumbers, the other dimension will cover all the spatial locations along the line focus. In this manner, data acquisition is sped up significantly. The same strategy is also used in Fiber Array Raman imaging[158, 159], in which a 2-D array of optical fibers on the sample end is rearranged to a 1-D array on the detector end for fast spectral acquisition. However, the spatial resolution is limited by the fiber size and the number of pixels are restricted by the number of fibers that could be mapped onto the CCD.

Another possible solution is to acquire Raman images at only a few wavenumbers corresponding to selected Raman peaks to speed up data acquisition. However, this could lead to inaccurate data interpretation due to insufficient information. This problem becomes more severe when selected Raman peaks are shared by multiple biochemical components[160]. Therefore, it is critical to develop a cost-effective and fast Raman acquisition method, which is compatible with the imaging configuration, without sacrificing the spectral resolution.

Reconstruction of optical spectra from a few narrow-band measurements is one potential method to solve this problem. In this method, optical images are acquired with a few bandpass filters instead of all wavelengths of interest thus data acquisition is much faster. The full optical spectrum at each pixel in the images can be

reconstructed from the corresponding narrow-band measurements. Quantitative studies have been carried out previously to recover reflectance spectra from a few narrow-band measurements using various methods, e.g. finite-dimensional modeling[68] and Wiener estimation[66]. Among those methods, Wiener estimation is one of most frequently used techniques because of its advantage in speed[161]. Several methods based on Wiener estimation, e.g. adaptive Wiener estimation[161] and modified Wiener estimation[92], have been developed for improving the reconstruction accuracy. However, there has been no previous effort in the use of Wiener estimation or similar methods for the reconstruction of Raman spectra to our best knowledge.

In this chapter, we demonstrated that traditional Wiener estimation and modified Wiener estimation can be used to reconstruct the full Raman spectrum from a couple of narrow-band Raman measurements. The two methods were evaluated on Raman spectra collected from live, apoptotic and necrotic leukemia cells with several different types of band-pass filter choices. Both Raman spectra with and without fluorescence background were tested, i.e. narrow-band measurements were synthesized from the Raman spectra before or after fluorescence background removal. The relative root mean square error (RMSE) of the reconstructed Raman spectrum relative to the measured Raman spectrum was averaged for all measurements to quantify the accuracy of reconstruction. Moreover, reconstructed Raman spectra were used to classify cells in terms of the death mode, i.e. apoptosis or necrosis, and the classification accuracy was compared to that using the measured Raman spectra.

6.2 Materials and Methods

6.2.1 Measurements of Raman Spectra

Raman spectra used in this study were measured from human chronic myelogenous leukemia cells (K562 cell line), which were purchased from American Type Culture Collection (Manassas, VA, US). The cells were divided into three groups. Two groups of cells were treated with different drugs to induce apoptosis and necrosis. The third group of cells was left untreated and served as the control group.

All spectra were acquired by a micro-Raman system (inVia, Renishaw, UK) coupled to a microscope (Alpha 300, WITec, Germany) in a backscattering geometry with a spectral resolution of 2 cm^{-1} . Ten cell spectra from each group were measured over a range of 600 cm^{-1} to 1800 cm^{-1} with an excitation wavelength of 785 nm generated by a diode laser. The details of cell preparation and measurements have been described elsewhere[160]. The raw Raman spectra are shown in Fig. 6.1.

Fig. 6. 1 The raw Raman spectra of the cells

6.2.2 Spectral Pre-processing and Simulated Narrow-band Measurements

The narrow-band measurement for a given bandpass filter was simulated by calculating the inner product of each original Raman spectrum or normalized Raman spectrum after fluorescence removal and the transmittance spectrum of the bandpass filter at the wavenumbers of interest. Narrow-band measurements synthesized from normalized Raman spectrum after fluorescence removal were used to get the results for Raman spectra in the absence of fluorescence background, while narrow-band measurements synthesized from original Raman spectra before fluorescence removal were used to generate the results for Raman spectra in the presence of fluorescence background. The fluorescence background in each spectrum was estimated by fitting the spectrum to the fifth order polynomial[162, 163] and subtracted from the spectrum. Then, each spectrum was smoothed by 5-point smoothing algorithm and normalized by dividing each data point by the maximum intensity in each spectrum. All data processing was coded and run in Matlab (Version 7.6, MathWorks, Natick, MA, US). The filter sets investigated in this study included Gaussian filters, commercial filters, Principle Components (PCs) based filters and non-negative PCs based filters. They were defined as follows.

6.2.2.1 Gaussian Filters

The transmittance spectrum of a Gaussian filter can be described by a Gaussian function with a specified central wavelength and standard deviation as shown in Eq. (6.1) below, which represents a set of simplified theoretical filters.

$$T(\lambda) = e^{\frac{-(\lambda-\mu)^2}{2\sigma^2}} \quad (6.1)$$

where $T(\lambda)$ represents the transmittance at the wavelength λ , μ is the central wavelength and σ is the standard deviation. Note that σ is related to the FWHM (full width at half maximum) of the filter but is not exactly the same value.

A total of 72 different Gaussian filters were investigated, in which each Gaussian filter is defined by the Gaussian function with the central wavelength varying from 830 nm to 910 nm with an increment of 10 nm and the standard deviation varying from 2.5 nm to 20 nm with an increment of 2.5 nm. Then the unit of the x-axis in the transmittance spectra was converted from wavelength (nm) to wavenumber (cm^{-1}) according to Eq. (6.2).

$$\Delta w = \left(\frac{1}{\lambda_0} - \frac{1}{\lambda_R} \right) \times 10^7 \quad (6.2)$$

where λ_0 is the excitation wavelength, λ_R is the Raman wavelength, Δw is the corresponding wavenumber.

6.2.2.2 Commercial Filters

A total of 37 commercial filters from five companies were selected because their transmittance spectra partially or totally overlap with the Raman spectra measured from cells. These filters included 2 bandpass emission filters (D850/20m, D850/40m) from Chroma Technique Cooperation (Bellows Falls, VT, US), 2 bandpass interference filters (NT84-790, NT84-791) from Edmund Optics (Barrington, NJ, US), 2 longpass filters (3RD850LP, 3RD900LP), 4 bandpass filters (XB142, XB143,

XB146, XB149), 1 emission filter (XF3308) and 4 laser line filters (XL19, XL40, XLK18, XLK20) from Omega Filters (Brattleboro, VT, US), 6 bandpass filters (FF01-830/2-25, FF01-832/37-25, FF01-835/70-25, FF01-840/12-25, FF01-910/5-25) from Semrock (Rochester, NY, US), 11 bandpass filters (FB830-10, FB840-10, FB850-10, FB850-40, FB860-10, FB870-10, FB880-10, FB880-40, FB890-10, FB900-40) and 5 laser line filters (FL830-10, FL850-10, FL880-10, FL905-25) from Thorlabs (Newton, New Jersey, US). This group of filters is readily available.

6.2.2.3 Principle Components (PCs) Based Filters

The transmittance spectra of PCs based filters are equivalent to the first a few PCs of the preprocessed Raman spectra. The transmittance spectra of non-negative PCs based filters were obtained in the same way using a published method[164]. Because the first a few PCs account for most variance contained in the preprocessed Raman spectra, i.e. they carry maximum information about the Raman spectra, PCs based filter set represent a theoretical set of filters that may yield the best performance. Nevertheless, PCs based filters could be fabricated or created using published methods [165, 166].

6.2.3 Estimation Methods

6.2.3.1 Traditional Wiener Estimation

Assume the preprocessed spectrum can be written as s and s is an $n \times 1$ matrix, in which n is the number of wavenumbers in each spectrum. The transmittance spectra of a filter set are represented by F . F is an $m \times n$ matrix, in which m is the number of

filters in the set. The noise in narrow-band measurements is denoted by e that is an $m \times 1$ matrix. Then the corresponding narrow-band measurements of the spectrum S generated by applying the filter set F is denoted by c , which can be expressed as

$$c = Fs + e \quad (6.3)$$

where c is an $m \times 1$ matrix. In Wiener estimation, a Wiener matrix W ($n \times m$ matrix) is used to transform narrow-band measurements c ($m \times 1$ matrix) into the corresponding Raman spectrum $\check{s}$ ($n \times 1$ matrix),

$$\check{s} = Wc \quad (6.4)$$

so that the mean square error between the original and estimated spectra is minimized.

The Wiener matrix W is given [86] by Eq. (6.5).

$$W = K_s F^T (F K_s F^T + K_e)^{-1} \quad (6.5)$$

where

$$K_s = E[ss^T], \quad K_e = E[ee^T] \quad (6.6)$$

In Eq. (6.5) and (6.6), the superscript “ T ” represents matrix transpose, the superscript “ -1 ” represents matrix inverse and $E[\cdot]$ represents an ensemble average. Plugging Eq. (6.6) into Eq. (6.5) and ignoring the noise term yields

$$W = E[sc^T][E[cc^T]]^{-1} \quad (6.7)$$

Once Wiener matrix W is created in the calibration stage as shown in Fig. 6.2, it can be applied to narrow-band measurements in the test stage to reconstruct the corresponding full Raman spectrum using Eq. (6.4).

Fig. 6. 2 General procedure of Wiener estimation

6.2.3.2 Modified Wiener Estimation

The method of modified Wiener estimation developed previously by our group, which more details can be found in Chapter 2, improves over traditional Wiener estimation by synthesizing new narrow-band measurements in the calibration step. In contrast to the traditional Wiener estimation, the modified Wiener estimation involves one post-processing step in addition to the calibration stage and the test stage, which are briefly reiterated below.

In the calibration stage, new reference and estimated narrow-band measurements, are synthesized from the known and estimated full optical spectra by an additional set of absorption filters. The estimated spectra are reconstructed by traditional Wiener estimation with original narrow-band measurements. Moreover, the relations for narrow-band correction can be found by using the following two strategies: (A) to model each reference narrow-band measurements as a second-order polynomial function of estimated narrow-band measurements; (B) to record the differences in each narrow-band

measurements between two sets. The modified Wiener matrix, which is created by the combination of the synthesized reference narrow-band measurements and original narrow-band measurements, is computed for the reconstruction of Raman spectra.

In the test stage, new narrow-band measurements are synthesized and corrected by the relations for narrow-band correction obtained in the calibration stage. Then, the corrected new narrow-band measurements and the original narrow-band measurements can be jointly used to reconstruct the Raman spectra accurately by applying the modified Wiener matrix. The modified Wiener estimation is more accurate than the traditional Wiener estimation because of the additional information provided by the synthesized narrow-band measurements.

Because two relations for narrow-band correction yield different estimated Raman spectra, a simple selection step is used to find the more accurate result of the two. The two sets of reconstructed Raman spectra are multiplied by the original system matrix to find the estimated narrow-band measurements. The set that yields narrow-band measurements closer to the original values will be selected as the final result.

6.2.3.3 General Procedure of Wiener Estimation

Both traditional Wiener estimation and modified Wiener estimation follow the same general procedure, which involve two stages, i.e. the calibration stage and the test stage. In the calibration stage, both measured Raman spectra and the corresponding narrow-band measurements are used to create a Wiener matrix. In the test stage, the Wiener matrix is employed to transform narrow-band measurements measured from

test samples to the corresponding Raman spectrum.

6.2.4 Relative RMSE and Classification Accuracy

Both relative RMSE and classification accuracy are used as the criteria to characterize the performance of Raman reconstruction. Relative RMSE is a frequently used criterion to measure the difference between the reconstructed values and actually observed values[167], which is defined in Eq. (6.8) below in this study.

$$\text{Relative RMSE} = \left[\frac{\sum_{i=1}^N [R_r(\lambda_i) - R_m(\lambda_i)]^2}{N \times \max[R_m(\lambda_i)]^2} \right]^{1/2} \quad (6.8)$$

where R_r and R_m are the reconstructed Raman spectrum and the measured Raman spectrum (both after fluorescence background removed), respectively, λ_i is the i -th wavenumber (i is varied from 1 to N) and the function, $\max[]$, returns the maximum intensity of the input spectrum.

A method of multivariate data analysis was used to perform the classification of cell death modes[160]. First, the first three principle components were calculated by applying principle component analysis method to the Raman spectra. Then, a method of linear discriminant analysis (LDA) was built to classify Raman spectra using the scores of the first three principle components. The classification accuracy was calculated as follows.

$$\text{Classification accuracy} = \frac{\text{number of correctly classified spectra}}{\text{Total number of spectra}} \times 100\% \quad (6.9)$$

The classification accuracies obtained using both the preprocessed and reconstructed Raman spectra were compared to each other to quantify the diagnostic value of reconstructed Raman spectra.

6.3. Results and Discussions for Raman Spectra in the Absence of Fluorescence Background

6.3.1 Representative Reconstructed Raman Spectra and Filters

Figure 6.3 shows the preprocessed Raman spectra, the Raman spectra reconstructed by traditional Wiener estimation using three commercial filters in the best case, the typical case and the worst case and the corresponding transmittance spectra of the filters. The typical case means that the relative RMSE of the reconstructed Raman spectrum is close to the mean value. Note that these three filters were the best among the combination of any three filters out of 37 commercial filters mentioned in the methods section, which are a laser line filter (XL19, Omega Optical), a BrightLine® single-band bandpass filter (FF01-857/30-25, Semrock) and a laser line filter (FL905-10, Thorlabs). The RMSEs in the best case, the typical case and the worst case were 0.0126, 0.0258 and 0.0536, respectively. Most Raman peaks were accurately recovered in the typical case. It is interesting to note that these three filters did not cover the entire spectrum of interest as normally expected, which will be discussed further later in the filter selection section.

Fig. 6.3 Comparison between the preprocessed Raman spectrum and the Raman spectrum reconstructed by traditional Wiener estimation with the best combination of three commercial filters in (a) the best case, (b) the typical case, (c) the worst case. (d) shows the filters' transmittance spectra.

Figure 6.4 shows the preprocessed Raman spectra, the Raman spectra reconstructed by traditional Wiener estimation using three non-negative PCs based filters in the best case, the typical case and the worst case and the corresponding transmittance spectra of the filters. The relative RMSEs in the best case, the typical case and the worst case are 0.0121, 0.0247 and 0.0502, respectively. It is interesting to see that the shapes of the transmittance spectra for these non-negative PCs based filters are similar to each other and they all resemble those of the preprocessed Raman spectra in the locations of spectral peaks. This is because the 2nd and 3rd non-negative PCs filters were created by summing the corresponding PC and the 1st PC of the original spectrum[164]. It is well known that the 1st PC resembles the mean spectrum.

Fig. 6. 4 Comparison between the preprocessed Raman spectrum and the Raman spectrum reconstructed by traditional Wiener estimation with three non-negative PCs based filters in (a) the best case, (b) the typical case, (c) the worst case. (d) shows the filters' transmittance spectra.

6.3.2 Effects of the Estimation method, the Filter set and the Number of Filters on the Accuracy of Reconstruction

Table 6.1 shows the comparison in the mean relative RMSE and the corresponding classification accuracy among Gaussian filters, commercial filters, PCs based filters and non-negative PCs based filters for both traditional Wiener estimation and modified Wiener estimation. Note that only three filters were used in each set. The Gaussian filters shown in Table 6.1 were described by Gaussian functions with central wavelengths of 870 nm, 870 nm and 900 nm, and standard deviations of 5 nm, 20 nm and 5 nm. The best commercial filters used here have been described in Figure 6.2. The classification accuracy achieved using the preprocessed Raman spectra was also shown for comparison.

Table 6. 1 Comparison in the best mean relative RMSE and the corresponding classification accuracy of Gaussian filters, commercial filters, PCs based filters and non-negative PCs based filters by both traditional Wiener estimation and modified

Wiener estimation ^a					
	Gaussian filters	Commercial filters	PCs based filters	Non-negative PCs based filters	Preprocessed Raman spectra
Mean relative RMSE by Traditional WE	2.63×10^{-2}	2.62×10^{-2}	2.47×10^{-2}	2.47×10^{-2}	0
Classification accuracy	86.67%	93.33%	93.33%	93.33%	93.33%
Mean relative RMSE by Modified WE	2.57×10^{-2}	2.53×10^{-2}	2.46×10^{-2}	2.39×10^{-2}	0
Classification accuracy	86.67%	93.33%	93.33%	93.33%	93.33%

^aWE is the acronym of Wiener Estimation

According to the mean relative RMSE, PCs based filters and non-negative PCs based filters showed the best performance. This could be attributed to the fact that, by definition, PCs based filters and non-negative PCs based filters captured most variance, i.e. information, in the original Raman spectra. Compared to the traditional Wiener estimation, the modified Wiener estimation method reduces the mean relative RMSE by 2.3%, 3.4%, 0.4% and 3.2%, respectively, when Gaussian filters, commercial filters, PCs based filters and non-negative PCs based filters were employed. Interestingly the reduction in the mean relative RMSE did not translate to the improvement in the classification accuracy. The classification accuracies achieved using the modified Wiener estimation were equal to those achieved using the traditional Wiener estimation, which implies that the reduction in relative RMSE was not large enough to change the classification result. In a large set of spectra, it would

be expected that a reduction in relative RMSE will lead to a monotonic increase in classification accuracy. For commercial filters, PCs based filters and non-negative PCs based filters, the classification accuracy obtained using both estimation methods were equal to that obtained using the preprocessed Raman spectra. This suggests that the reconstructed spectra obtained in these cases were equivalent to the original Raman spectra in terms of the amount of information retained in reconstruction for classification.

Table 6. 2 Comparison in the best mean relative RMSE and corresponding classification accuracy of Gaussian filters, commercial filters, PCs based filters and non-negative PCs based filters using both three filters and six filters by traditional

Wiener estimation					
	Gaussian filters	Commercial filters	PCs based filters	Non-negative PCs based filters	Preprocessed Raman spectra
Mean relative RMSE with 3 filters	2.63×10^{-2}	2.62×10^{-2}	2.47×10^{-2}	2.47×10^{-2}	0
Classification accuracy	86.67%	93.33%	93.33%	93.33%	93.33%
Mean relative RMSE with 6 filters	2.18×10^{-2}	2.15×10^{-2}	2.01×10^{-2}	2.01×10^{-2}	0
Classification accuracy	86.67%	90%	93.33%	93.33%	93.33%

Table 6.2 shows the comparison in the mean relative RMSE and classification accuracy obtained using three and six Gaussian filters, commercial filters, PCs based filters and non-negative PCs based filters for traditional Wiener estimation. The choices of three filters in each filter set were the same as in Table 6.1. The choices of six filters were made as follows. The Gaussian filters have central wavelengths of 850 nm, 860 nm, 870 nm, 870 nm, 880 nm and 900 nm and standard deviations of 2.5 nm,

15nm, 5 nm, 20nm, 2.5 nm and 5 nm. The global search for the best combination of 6 Gaussian filters was impractical due to a huge number of possible filter configurations (about 15.6 million in total). Therefore, we fixed the best three filters identified in Table 1 and searched for the additional three filters to obtain a suboptimal combination of 6 filters. The best combination of six commercial filters were found in the same manner due to the same reason. The identified additional commercial filters included two bandpass filters (FB850-10 and FB880-40, Thorlabs) and a laser line filter (XLK20, Omega Filters). For PCs based filters and non-negative PCs based filters, three additional filters were just simply defined by the next three principal components.

It is clear that using six filters always yielded significant reductions in the mean relative RMSE, which were 17.1%, 17.9%, 18.6% and 18.6% for Gaussian filters, commercial filters, PCs based filters and non-negative PCs based filters, respectively. This is because a larger number of narrow-band measurements retained more information carried by the original spectra, which was used in spectral reconstruction. Similar to the case of three filters, the PCs based filters and non-negative PCs based filters always showed the smallest relative RMSE in the case of six filters.

Similar to Table 6.1, the reduction in the mean relative RMSE through the employment of additional filters did not result in improvement in the classification accuracy because three optimal filters have yielded the best classification accuracy possible even with the perfect reconstruction. The classification accuracy in case of three Gaussian filters and six Gaussian filters were lower than other cases. Moreover,

there was no improvement or a decrease in the classification accuracy with six commercial filters compared to three commercial filters for these two sets of filters, although the mean relative RMSE in case of six filters was smaller. This seemingly conflicting result is likely due to the fact that the mean relative RMSE only shows the similarity between the reconstructed Raman spectra and the original spectra whereas the classification accuracy was determined by the differences in the Raman spectra across cell groups. Therefore it is possible that the additional filters minimized the difference between the reconstructed Raman spectra and the original spectra in all cell groups, but the differences in reconstructed Raman spectra across cell groups were artificially reduced in the process of reconstruction.

Among those different types of filters, Gaussian filters are perhaps the most cost effective ones due to the simplicity in transmittance spectra if they are readily available, such as FL905-10 in Fig. 6.2(d), compared with those commercial filters with sharp edges in transmittance spectra, such as FF01-857/30-25 in Fig. 6.2(d), and PCs based filters and non-negative PCs based filters. According to the mean relative RMSE in Tables 6.1 and 6.2, Gaussian filters showed similar mean relative RMSE as commercial filters. However, its classification was lower than the other types of filters, which may be due to the unchanged amplitude of the transmittance spectra in Gaussian filters in this study. Although PCs based filters and non-negative PCs based filters give the best performance in both mean relative RMSE and classification accuracy, those filters can be expensive and complicated to fabricate although there are published methods to make them [168, 169].

6.3.3 Rules of Thumb for Commercial Filter Selection

Upon the analysis of the optimal combination of three filters out of 37 commercial filters, the following rules of thumb have been identified, which could guide the choices of commercial filters for optimal spectral reconstruction in the future. The first rule is that the transmittance spectra of the filters should cover the entire range of spectra especially those important spectral regions such as Raman peak locations.

Fig. 6. 5 Comparison of the (a) variances of the original Raman spectra at all wavenumbers (b) the transmittance spectra of the best combination and (c) the transmittance spectra of the worst combination of three filters out of 37 commercial filters

The second rule is that the wavelength regions with larger variances across samples are more important than those with smaller variances. The spectra in such important

wavelength regions should be acquired by filters with higher transmittance, so that more information can be retained in narrow-band measurements. This rule is evident in the comparison between the variance of the preprocessed Raman spectra at all wavenumbers and the transmittance spectra for the best and worst commercial filter sets as shown in Figure 6.5. In this figure, the largest variances are located at 1300 cm^{-1} and 1680 cm^{-1} . The transmittance spectra of the best combination of three commercial filters cover both variance peaks. In contrast, the transmittance spectra of the worst combination of three commercial filters do not cover either of the two.

6.4. Results and Discussions for Raman Spectra in the Presence of Fluorescence Background

Table 6.3 Comparison of relative RMSE of reconstructed Raman spectra after removing the background from the wide-band measurements using different types and

numbers of filters

	Gaussian filters	Commercial filters	PCs based filters	Non-negative PCs based filters
Relative RMSE with 3 filters	4.85×10^{-2}	5.17×10^{-2}	6.93×10^{-2}	6.93×10^{-2}
Relative RMSE with 4 filters	4.28×10^{-2}	4.22×10^{-2}	5.83×10^{-2}	5.83×10^{-2}
Relative RMSE with 5 filters	3.60×10^{-2}	3.50×10^{-2}	3.20×10^{-2}	3.20×10^{-2}
Relative RMSE with 6 filters	3.00×10^{-2}	2.93×10^{-2}	2.57×10^{-2}	2.57×10^{-2}

Table 6.3 shows the comparison of relative RMSE of reconstructed Raman spectra after removing the background from the wide-band measurements using different types and numbers of filters. The improvements from 3 to 4 filters are 11.8%, 18.4%, 15.9% and 15.9% for Gaussian filters, commercial filters, PCs based filters and non-negative PCs based filters respectively. The improvements from 4 to 5 filters are

15.9%, 17.1%, 45.1% and 45.1% and the improvements from 5 to 6 filters are 16.7%, 16.3%, 19.7% and 19.7%. Fig. 6.6 shows the comparison between the preprocessed Raman spectrum and the Raman spectrum reconstructed by Wiener estimation with the best combination of six commercial filters, i.e. NT84-791, FB860-10, FB850-10, FB880-10, FB900-40 and FL850-10. The typical case is the reconstructed Raman spectrum with relative RMSE similar to the mean relative RMSE, while the best case and worst case is the reconstructed Raman spectrum with minimum relative RMSE and maximum relative RMSE. The relative RMSEs are 0.0102, 0.0295, 0.0564 for best case, typical case and worst case, respectively. Fig. 6.7 shows the comparison between the preprocessed Raman spectrum and the Raman spectrum reconstructed by Wiener estimation with the best combination of six non-negative PCs based filters. The relative RMSEs are 0.0093, 0.0255, 0.0499 for best case, typical case and worst case, respectively.

Fig. 6. 6 Comparison between the preprocessed Raman spectrum and the Raman spectrum reconstructed by Wiener estimation with the best combination of six commercial filters in (a) the best case, (b) the typical case, (c) the worst case. (d) shows the filters' transmittance spectra.

Fig. 6. 7 Comparison between the preprocessed Raman spectrum and the Raman spectrum reconstructed by Wiener estimation with the best combination of six non-negative PCs based filters in (a) the best case, (b) the typical case, (c) the worst case. (d) shows the filters' transmittance spectra

For Raman spectra, the reconstruction accuracies with Gaussian filters and commercial filters are better than PCs based filters for 3 and 4 filters. This is because 3 and 4 PCs based filters capture more information from the fluorescence background and less from the Raman signal. PCs based filters are able to capture the most variance, i.e. information, from original spectra by its definition. The fluorescence background in the Raman spectra show larger variance compared with the Raman signal. And PCs based filters show better reconstruction accuracies than Gaussian filters and commercial filters for 5 and 6 filters. And the improvement for PCs based filters from 4 to 5 filters is significant, which means more Raman information is

collected based on the sufficient information collected from the 3 PCs based filters. We also test the PCs based filters that are generated only from the Raman signal or fluorescence background. For PCs based filters from Raman signal, the relative RMSEs are 1.43×10^{-1} , 1.43×10^{-1} , 5.60×10^{-2} and 5.42×10^{-2} for 3, 4, 5 and 6 PCs based filters, respectively. For PCs based filters from fluorescence background, the relative RMSEs are 8.86×10^{-2} , 8.28×10^{-2} , 9.69×10^{-2} and 8.12×10^{-2} for 3, 4, 5 and 6 PCs based filters, respectively. The reconstruction accuracies are much worse than the PCs based filters from Raman spectra with fluorescence background, which indicates that information from both Raman signal and fluorescence background is important during the reconstruction. What's more, additional filters can improve the reconstruction accuracy significantly. And the PCs based filters show better result for 5 and 6 filters compared with Gaussian filters and commercial filters due to the ability of capture more information from both Raman signal and fluorescence background. Compared with Raman spectra without fluorescence background in section 6.3, the Raman spectra with fluorescence background show worse performance, which indicates that the higher signal to noise will improve the reconstruction accuracy significantly. Therefore, minimization of the background or enhance the Raman signal will further improve the reconstruction accuracy for this method.

We anticipate that this method of reconstruction will be advantageous when employed in Raman imaging. Currently most Raman imaging techniques use point scanning or line scanning, in which every scan would involve the acquisition of Raman intensity at many wavenumbers. However, this way will yield Raman images with high

spectral resolution but low spatial resolution. Wide-field Raman imaging using a CCD could be performed at each wavenumber, this would require a filter with an extremely narrow pass band and tunable central wavelength or a number of filters with different central wavelengths. Moreover, it would be very time consuming given the number of wavenumbers involved (hundreds to thousands depending on spectral resolution required). Our Raman imaging approach consists of two steps, i.e. taking the narrow-band Raman images and then performing spectral reconstruction at each pixel of the Raman image. Therefore, our method requires only a few narrow-band filters with much larger bandwidths to get a few Raman images and the full Raman spectrum at each pixel can be reconstructed. The potential improvement in the speed will be dramatic just considering the difference in the number of Raman images required between traditional Raman imaging and the proposed strategy. The narrow-band Raman imaging system can be simply implemented by taking narrow-band Raman images sequentially by using a filter wheel loaded with different narrow-band filters. We are also developing a Raman imaging system to acquire multiple narrow-band images simultaneously.

6.5. Conclusion

In conclusion, we have developed a fast method for the reconstruction of Raman spectra based on Wiener estimation from a few narrow-band measurements. According to the mean relative RMSE, the agreement between the reconstructed spectra and the original spectra were excellent; furthermore, reconstructed spectra

retained most biochemical information important for sample classification in terms of the accuracy of sample classification using reconstructed spectra. The method of modified Wiener estimation previously developed by our group showed moderate improvement over traditional Wiener estimation. Additional filters can improve the mean RMSE significantly. However, no improvement in the classification accuracy was observed for most filter sets when using either modified Wiener estimation or additional filters. It is also found that the classification accuracies using reconstructed spectra obtained by applying the best combination of commercial filters, the PCs based filters and the non-negative PCs based filters are identical to that using the original Raman spectra. Two rules of thumb were identified to guide the choices of commercial filters for optimal reconstruction, which is to choose filters that cover the entire spectral range and maximize the transmittance in spectral regions that exhibit the highest variance in the original Raman spectra. In addition, the reconstruction of Raman spectra in the presence of fluorescence background shows worse performance compared with the Raman spectra in the absence of fluorescence background. However, the Raman spectroscopy will be further simplified due to the employment of the narrow-band measurements from the Raman spectra with fluorescence background. Given the excellent reconstruction accuracy and the fast acquisition in narrow-band Raman measurements, we believe that this approach represents a promising new direction for Raman spectroscopy in an imaging setup to investigate fast changing phenomena in biological samples.

Chapter 7: Recovery of Raman Spectra from Measurements With Low Signal to Noise Ratio

[Copyright permission from: S. Chen et. al., Recovery of Raman spectra with low signal-to-noise ratio using Wiener estimation, Opt Express 22(10), 12102 (2014).]

7.1 Introduction

Besides the Raman reconstruction ability in Chapter 6, the proposed methods has the advantage to improve the signal to noise ratio due to the integration along the wavenumber dimension. In this Chapter, the proposed method was investigated to recover the Raman spectra from the measurements with low SNR, which compete with four commonly used de-noising methods.

Raman spectroscopy is a laser-based spectroscopic technique that exploits Raman scattering for qualitative or quantitative biological material characterization [160]. Rich biochemical information can be revealed from resulting Raman shifts, which depend on the specific vibrational modes of molecules in tissues and cells [170]. Therefore, Raman spectra or peaks inside could be employed to differentiate biological components. This method has shown great potential in many biomedical applications [171, 172]. Unfortunately, such applications are often hampered by inherently weak Raman signals from biological molecules [173]. In this case, measurement noises obscure Raman peaks of interest rendering a low signal-to-noise ratio (SNR) [174]. It is common to solve this problem by increasing the power of the excitation laser and/or exposure time. However, these methods cannot be used when

measuring unstable materials or observing fast changing phenomena. Therefore, it is important to develop a method to quickly recover Raman spectra with low SNR without increasing laser power.

Smoothing and filtering are two common categories of de-noising methods in Raman spectroscopy [175]. Savitzky-Golay (SG) algorithm is one of the most frequently used smoothing methods to de-noise Raman spectra [176], in which each segment of the original Raman spectrum in a small window is smoothed by fitting it to a polynomial function [177]. When the window size is small, the smoothing outcome will be poor. When the window size is large, the spectral resolution will degrade and those weak spectral features will be distorted. A tradeoff needs to be made between the smoothing outcome and the spectral resolution by appropriately choosing the window size and the order of the polynomial function. In contrast, finite impulse response (FIR) filtration [175], wavelet transform [178, 179] and factor analysis [180], are commonly used filtering techniques for Raman de-noising. FIR filtration offers excellent preservation of the spectral shape, but it is demanding in computation. In the technique of wavelet transform, spectral data are decomposed into the wavelet domain and reconstructed after thresholding for noise removal. Currently, the selection of wavelet filters, threshold and other parameters in wavelet transform is strongly problem dependent [179]. In factor analysis, acquired spectra are projected into the orthonormal set of subspectra by singular value decomposition. The original spectral information is maintained by the linear combination of optimized number of subspectra with large singular values. The subspectra with small singular values

(usually below 0.5% of the maximal value) are treated as white noise [181]. The method will lose the ability to decompose the signal and the noise when the SNR is low, because the noise would have comparable or even more contribution to the acquired spectra compared to the signal.

In our previous study in Chapter 6[113], we have successfully reconstructed the Raman spectra of cells and biological fluid from narrow-band measurements using the WE method without sacrificing spectral resolution. In this study, we propose the reconstruction of Raman spectra from narrow-band measurements based on Wiener estimation (WE) method as an alternative method to denoise Raman spectra with low SNR. By synthesizing narrow-band measurements from low-SNR spectra, the integration along the wavenumber dimension in the synthesis reduces the effect of noise. Then the narrow-band measurements are used to reconstruct high-SNR spectra based on Wiener estimation. To our best knowledge, this is the first time that spectral reconstruction involving Wiener estimation is used to recover the high-SNR Raman spectra from narrow-band measurements that were synthesized from low-SNR Raman measurements. Although traditional Wiener filtration for noise reduction and spectral reconstruction based on Wiener estimation have been performed separately before, our method is different from either one of them. In traditional Wiener filtration, narrow-band measurements are never synthesized and the Wiener filter is directly applied to a signal with the additive noise. In the traditional spectral reconstruction based on Wiener estimation, there is no previous study in which narrow-band measurements are synthesized from low-SNR Raman spectra to recover the

corresponding high-SNR Raman spectra. So the traditional spectral reconstruction based on Wiener estimation is not able to de-noise the spectra.

More specifically, we systematically investigated the recovery of Raman spectra from Raman measurements with low SNR obtained by using a series of short exposure time values during spectral acquisition, in which Raman spectra with high SNR obtained using a long exposure time served as the reference measurement for comparison. Narrow-band measurements synthesized from Raman spectra with low SNR using a set of non-negative principal components (PCs) based filters were used to reconstruct Raman spectra with high SNR by Wiener estimation. The choice of non-negative PCs based filters in the process ensures that most variance contained in the original Raman measurements are retained. The method was validated on Raman spectra measured from 25 phantoms with two different Raman scatterers, i.e. urea and potassium formate, at different concentrations and 20 Raman spectra measured from bacteria samples. Four commonly used Raman de-noising methods, i.e. SG algorithm, FIR filtration, wavelet transform and factor analysis, were evaluated on the same sets of Raman spectra for comparison. According to the results, the agreement between the Raman spectra recovered by WE method and the reference Raman spectra was significantly better than the four common de-noising methods. Therefore our method represents an effective alternative to recover Raman spectra from samples with intrinsically low Raman efficiency or those acquired in short time from fast changing phenomena thus with low SNR.

7.2 Materials and Methods

7.2.1 Principle of Wiener Estimation for Recovery of Raman Spectra

Wiener estimation [66, 92] is used to recover Raman spectra from low-SNR Raman measurements and results are compared to the reference Raman spectra with high SNR. The low SNR Raman measurements are collected from a sample with short exposure time and single accumulation, while the corresponding reference Raman spectra with high SNR are collected from the same sample with long exposure time and multiple accumulations.

In the recovery process, there are two data sets involved, i.e. the calibration data set and test data set. The calibration data set contains both Raman measurements with low SNR and the corresponding reference Raman measurements with high SNR. In the test data set, only Raman measurements with low SNR are present.

The role of the calibration data set is to yield the Wiener matrix [86] when ignoring noise term according to Eq. (7.1) below.

$$W = E(S_{high} C_{cal}^T) [E(C_{cal} C_{cal}^T)]^{-1} \quad (7.1)$$

where W is the Wiener matrix, S_{high} is the reference Raman measurements with high SNR and C_{cal} is the narrow-band measurements synthesized from Raman measurements with low SNR in the calibration data set.

Narrow-band measurements C_{cal} are synthesized from the Raman measurements with low SNR in the calibration data set using non-negative PCs based filters that were generated using a published method [164] according to Eq. (7.2) below.

$$C = FS_{low} \quad (7.2)$$

where C ($n \times 1$ matrix) is the synthesized narrow-band measurements, F ($n \times m$ matrix) is the transmission spectrum of non-negative PCs based filters and S_{low} ($m \times 1$ matrix) is the Raman spectrum with low SNR. Note that m is the number of discrete wavenumbers in the Raman spectrum and n is the number of filters in synthesized narrow-band measurements.

Then the recovery of Raman spectra from a set of Raman measurements with low SNR in the test data set is achieved according to Eq. (7.3) below.

$$\hat{S}_{high} = WC_{test} \quad (7.3)$$

where $\hat{S}_{high}$ ($m \times 1$) is the recovered Raman spectrum with high SNR and C_{test} is the narrow-band measurements synthesized according to Eq. (7.2) from Raman measurements with low SNR in the test data set.

The leave-one-out method [182] is used for cross validation in our study to fully utilize all samples in an unbiased manner. In this strategy, one sample is selected as the test data set and the rest of samples serve as the calibration data set. The procedure is repeated until all the samples have been tested.

7.2.2 Sample Preparation and Measurements

Two sets of samples were used to validate our method, which includes 25 phantom samples and 20 bacteria samples. The tunability in the composition of phantoms and the concentration of each Raman scatterer in the phantoms makes the phantom study ideal in quantitative evaluation. Bacteria samples were used because the Raman signal from bacteria is weak even with a long exposure time thus it is an excellent target to

demonstrate the effectiveness of the recovery of Raman spectra with low SNR using the proposed method.

The phantoms were made by mixing urea (V3171, Promega corporation, US) and potassium formate (294454-500G, Sigma-Aldrich, US) in 1.5% agar (PC0701-500G, Vivantis Technologies, US) dissolved in distilled water. The concentrations for both urea and potassium formate under investigation included 0.25 M, 0.5 M, 1 M, 1.5 M and 2 M. Raman spectra with both low SNR and high SNR were measured over a range from 600 cm^{-1} to 1800 cm^{-1} , by using a micro-Raman system (innoRam-785S, B&W TEK, US) coupled to a video microscope sampling system (BAC151A, B&W TEK, US). The excitation wavelength was 785 nm and the spectral resolution was 4 cm^{-1} . The exposure time for Raman spectra with low SNR was 50 ms and each spectrum was accumulated for once, while the exposure time for Raman spectra with high SNR was 10 s and each spectrum was accumulated for 30 times.

The bacterial samples, including *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 29213), was grown overnight in Tryptic Soy Agar Plates (TSA) at $35\text{ }^{\circ}\text{C}$. Few colonies were picked up and suspended in Phosphate Buffered Saline (PBS) to a concentration of 1×10^8 CFU/ml, which were then concentrated by centrifuging at 10,000 rpm for five minutes and the supernatant was discarded. After that, the bacteria sample was washed twice in 1 mL distilled water to remove any culture media and finally suspended in 100 μL distilled water. The suspended samples with a volume of 2 μL were repeatedly dropped at the same position on an aluminum foil for five or ten times to create rounded areas with

different bacteria concentrations. Five pairs of Raman spectra, one with low SNR and the other with high SNR in each pair, were measured over a range from 600 cm^{-1} to 1600 cm^{-1} from five different locations in each rounded area. Among these five locations picked randomly, three of them were located at the edge and two at the center of the drop in each rounded area to account for the variability in bacteria concentration. This procedure of sample preparation and Raman measurements were repeated for a total of four times to generate 20 pairs of Raman spectra. Raman measurements were performed using a micro-Raman system (inVia, Renishaw, UK) coupled to a microscope (Alpha 300, WITec, Germany) in a backscattering setup. The excitation wavelength was 633 nm and the spectral resolution was 2 cm^{-1} . The exposure time for Raman spectra with low SNR was 1 s and each spectrum was accumulated for once only, while the exposure time for Raman spectra with high SNR was 10 s and each spectrum was accumulated for 30 times. In addition, Raman spectra with an exposure time of 5 s or 10 s and accumulation of once were measured for comparison.

In order to quantify the noise level of the Raman spectra with low SNR in this study, the SNR was defined as follows in Eq. (7.4).

$$\text{SNR} = \frac{s}{\sigma} \quad (7.4)$$

where s is the largest peak intensity of the reference Raman spectrum (with high SNR) divided by a scaling coefficient and σ is the root mean square deviation between the low-SNR Raman spectrum and the scaled reference Raman spectrum in the entire spectral range. The scaling coefficient is obtained by dividing the sum of all the

intensity values in the reference Raman spectrum by that in the corresponding low-SNR Raman spectrum. Due to the significant pixel bias, which refers to the detector reading independent of exposure time [183], of the Raman system used in phantom experiments, pixel bias correction was conducted in phantom spectra before SNR calculation. Note that the pixel bias issue was insignificant in the Raman system used in bacteria measurements thus no correction was performed before SNR calculation for the bacteria data. The pixel bias value for any given phantom spectrum was extracted from the linear fitting between the sums of all intensity values in the reference Raman spectra and those in the corresponding low SNR Raman spectra. The traditional SNR is equivalent to the average peak height (usually above the baseline) divided by the standard deviation of the peak height [184]. The numerator, s , in Eq. (7.4) is the same as the average peak height in the traditional definition of SNR. The denominator of Eq. (7.4), σ , is equivalent to the standard deviation of the peak height in the traditional SNR because the peak intensity is nearly on the same level as the background intensities in low-SNR Raman measurements as shown in Fig. 7.1(a) and 7.2(a).

7.2.3 Evaluation of SG Algorithm, FIR Filtration, Wavelet Transform and Factor Analysis

Four de-noising methods were used to recover Raman spectra from low-SNR Raman measurements for comparison. Optimal accuracy was found by selecting the best combination of parameters. For SG algorithm, the frame size was varied from 3 to the

maximum odd number that was smaller than or equal to the number of data points in each spectrum. The polynomial degree was varied from 1 to 9. For FIR filtration, the window size was varied from 2 to an integer smaller than or equal to 1/3 of the number of data points in each spectrum and the cutoff frequency was ranged from 1×10^{-10} to 1. For wavelet transform, although there exist improved wavelet transform methods [178, 185], the relevant codes are not publically available. Therefore only the basic wavelet transform, which can be implemented using built-in functions in Matlab, was used. Common wavelet filters built in Matlab were tested and the level of decomposition was varied from 1 to 10. The thresholds were selected according to Birge-Massart strategy [186, 187] and both soft and hard thresholding were evaluated. For factor analysis, the number of subspectra used for linear combination was varied from 1 to the maximum value possible [180, 181].

The criterion used to define the accuracy of recovered Raman spectra in this study was the relative root mean square error (RMSE), which was formulated as in Eq. (7.5)

$$\text{Relative RMSE} = \left[\frac{\sum_{i=1}^N [R_{low}(\lambda_i) - R_{high}(\lambda_i)]^2}{N \times \{\max[R_{high}(\lambda_i)]\}^2} \right]^{1/2} \quad (7.5)$$

where R_{low} is the Raman signal (normalized with or without removing fluorescence background) reconstructed from the measured Raman spectrum S_{low} using Wiener estimation as shown in Eqs. (7.1)-(7.3), R_{high} is the Raman signal (normalized with or without removing fluorescence background) computed from the reference Raman signal S_{high} , λ_i is the i -th wavenumber (i is varied from 1 to N) and the function $\max[]$ returns the maximum intensity in the input spectrum. The fluorescence background

was removed by fitting the original spectrum to the fifth order polynomial and subtracting the fitted spectrum from the original one [163]. The spectrum was then normalized by dividing the intensity at each wavenumber by the sum of the intensities at all wavenumbers.

7.3 Results

Fig. 7.1 shows (a) Raman spectra with low SNR, (b) reference Raman spectra with high SNR measured from phantoms and (c) first three non-negative PCs based filters generated from the reference Raman spectra. Fig. 7.2 shows (a) Raman spectra with low SNR, (b) reference Raman spectra with high SNR measured from bacteria samples and (c) first six non-negative PCs based filters generated from the reference Raman spectra. It should be noted that these are raw spectra without going through background removal. For both phantoms and bacteria samples, the Raman spectra with low SNR are much noisier than reference Raman spectra and Raman peaks are overwhelmed by noise. The mean SNR of Raman spectra with low SNR for phantom samples is 6.02, while the mean SNR of Raman spectra with low SNR for bacteria samples is 0.98. Although the exposure time for bacteria samples (1 s) is longer than the exposure time for phantoms (50 ms), the bacteria samples show worse SNR due to the intrinsically weak Raman signal from bacteria.

Fig. 7. 1 (a) Raman spectra with low SNR and (b) reference Raman spectra with high SNR measured from 25 phantoms and (c) non-negative PCs based filters'

transmittance spectra

Fig. 7. 2 (a) Raman spectra with low SNR (b) reference Raman spectra with high SNR measured from bacteria samples and (c) non-negative PCs based filters' transmittance spectra

Table 7. 1 Comparison in the mean relative RMSE of Raman spectra of phantoms (after fluorescence background removal and normalization) recovered/smoothed from low-SNR Raman measurements using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method

	SG algorithm	FIR filtration	Wavelet transform	Factor analysis	WE method
Mean relative RMSE	1.07×10^{-1}	1.09×10^{-1}	1.02×10^{-1}	1.06×10^{-1}	1.99×10^{-2}

Table 7.1 shows the comparison in the mean relative RMSE of reconstructed/smoothed Raman spectra (after removing fluorescence background and normalization as described in the Materials and Methods section) from phantoms using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method. The mean relative RMSE for WE method is only 18.6%, 18.3%, 19.5% and 18.8% of those for SG algorithm, FIR filtration, wavelet transform and factor analysis, respectively. Fig. 7.3 shows the comparison of the reference Raman spectrum and the Raman spectra recovered/smoothed from low-SNR Raman measurements using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method in the typical case, in which the relative RMSE is close to the mean value.

Table 7. 2 Comparison in the mean relative RMSE of Raman spectra of bacteria samples (after fluorescence background removal and normalization)

recovered/smoothed from low-SNR Raman measurements using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method

	SG algorithm	FIR filtration	Wavelet transform	Factor analysis	WE method
Mean relative RMSE	1.47×10^{-1}	1.54×10^{-1}	1.45×10^{-1}	1.48×10^{-1}	8.21×10^{-2}

Fig. 7. 3 Comparison of the reference Raman spectra from phantoms and the

corresponding spectra recovered from low-SNR Raman measurements using (a) SG algorithm, (b) FIR filtration method, (c) wavelet transform, (d) factor analysis and (e) WE method, in which the relative RMSE is close to the mean value. Fluorescence background has been removed and spectra have been normalized.

Table 7.2 shows the comparison in the mean relative RMSE of Raman spectra (after fluorescence background removal and normalization) from bacteria samples recovered using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method. The mean relative RMSE for WE method is 55.9%, 53.3%, 56.6%, 55.5% those of SG algorithm, FIR filtration, wavelet transform and factor analysis, respectively. Fig. 7.4 shows the comparison of the reference Raman spectra and the corresponding spectra recovered/smoothed using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method, in which the relative RMSE is close to the mean value.

Fig. 7. 4 Comparison of the reference Raman spectra from bacteria samples and the Raman spectra recovered from low-SNR Raman measurements using (a) SG algorithm, (b) FIR filtration method, (c) wavelet transform, (d) factor analysis and (e) WE method, in which the relative RMSE is close to the mean value. Fluorescence background has been removed and spectra have been normalized.

Table 7. 3 Comparison in the mean relative RMSE of Raman spectra of phantoms (after normalization but without background removal) recovered/smoothed from low-SNR Raman measurements using SG algorithm, FIR filtration, wavelet transform,

factor analysis and WE method

	SG algorithm	FIR filtration	Wavelet transform	Factor analysis	WE method
Mean relative RMSE	8.83×10^{-2}	8.81×10^{-2}	8.83×10^{-2}	8.79×10^{-2}	2.05×10^{-2}

In order to clearly see whether the background removal process influences the results, the mean relative RMSE of recovered/smoothed Raman spectra (after normalization but without background removal), using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method, were compared as shown in Table 7.3. The mean relative RMSE for WE method is 23.2%, 23.3%, 23.2%, 23.3% those of SG algorithm, FIR filtration, wavelet transform and factor analysis, respectively. Table 7.4 shows the comparison in the mean relative RMSE of Raman spectra from bacteria samples (after normalization but without background removal) recovered using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method. The mean relative RMSE for WE method is 63.3%, 57.8%, 62.9%, 45.0% those of SG algorithm, FIR filtration, wavelet transform and factor analysis, respectively. In both Tables 7.3 and 7.4, WE method shows significant improvement compared with other techniques. Therefore, WE method shows much better performance on the raw Raman measurements as well when fluorescence background is not removed.

Table 7. 4 Comparison in the mean relative RMSE of Raman spectra of bacteria samples (after normalization but without background removal) recovered/smoothed from low-SNR Raman measurements using SG algorithm, FIR filtration, wavelet

transform, factor analysis and WE method

	SG algorithm	FIR filtration	Wavelet transform	Factor analysis	WE method
Mean relative RMSE	1.58×10^{-2}	1.73×10^{-2}	1.59×10^{-2}	2.22×10^{-2}	1.00×10^{-2}

7.4. Discussions

Results from phantom samples as in Fig. 7.3 show that the agreement in peak locations between reference Raman spectra and Raman spectra recovered from low-SNR Raman measurements using WE method as shown in Fig. 7.3(e) is excellent and spectral shape information is mostly preserved. In Raman spectra recovered using SG algorithm as shown in Fig. 7.3(a), peak locations are distorted and spectral shape information is lost, because those weak Raman features in spectra with extremely low SNR can be easily smoothed out. FIR method, as shown in Fig. 7.3(b), fails to distinguish Raman peaks from noise due to the large variance of the noise, thus it shows poor performance in the recovery of the peak locations and spectral shape. Wavelet transform, as shown in Fig. 7.3(c), shows good performance in noise removal, but important spectral shape information such as the central wavelengths and bandwidths of peaks is lost. The Raman spectra recovered using factor analysis are still noisy, as shown in Fig. 7.3(d), which suggests this method does not work well in this case. This is likely because the signal and the noise have comparable contributions to the measured Raman spectra.

Results from bacteria samples in Fig. 7.4 show that the agreement in the locations of

those peaks with relatively high intensity between reference Raman spectra and Raman spectra recovered from low-SNR Raman measurements using WE method as shown in Fig. 7.4(e) is excellent and the spectral shape information is mostly preserved. However, several small peaks are not recovered well, which is not as good as in the recovery of phantom spectra. This is because the Raman signals of bacteria samples are much weaker than those in phantoms. Compared with WE method as shown in Fig. 7.4(e), the SG algorithm, FIR filtration and wavelet transform, illustrated in Fig. 7.4(a-c), show much worse results, in which most peak locations are shifted and spectral shape is distorted. The Raman spectra recovered using factor analysis as shown in Fig. 7.4(d) are still noisy, which suggests this method does not work well in terms of noise removal.

In Fig. 7.3 and 7.4, it can be found that the reference spectrum is different for each noise reduction technique. The reason for such a difference in the reference spectra is explained as follows. The test results on a single phantom or bacteria sample was inadequate to represent the performance for all techniques because each technique gives different performance on various phantoms or bacteria samples. To avoid this issue, 25 phantoms and 20 bacteria samples were used and the mean relative RMSE was used to evaluate the overall performance of those techniques. In this sense, the typical cases, i.e. the relative RMSE close to the mean relative RMSE, can effectively represent the performance of different techniques. However, it is impossible to find a phantom or bacteria sample that was the typical case for all five different techniques, because each technique showed different performance on the same phantom or

bacteria. Therefore, five different phantoms or bacteria samples were selected, in which one represented the typical case for each technique.

For bacteria samples, we have tested the recovery of Raman spectra from two other sets of low-SNR measurements with exposure times of 5 s and 10 s in addition to an exposure time of 1 s. The SNRs are 2.09 and 2.87 for low-SNR measurements with exposure times of 5 s and 10 s, respectively. Table 7.5 shows the comparison in the mean relative RMSE of Raman spectra of bacteria samples (after fluorescence background removal and normalization) recovered from low-SNR Raman measurements (acquired with different exposure time) using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method. With an increasing exposure time, all methods, i.e. SG algorithm, FIR method, wavelet transform, factor analysis and WE method, yield better performance. WE method always yields the best result among the five methods, while SG algorithm, FIR filtration, wavelet transform and factor analysis show performance similar to each other but considerably worse than WE method.

Table 7. 5 Comparison in the mean relative RMSE of Raman spectra of bacteria samples (after fluorescence background removal and normalization) recovered from low-SNR Raman measurements with different exposure time using SG algorithm, FIR filtration, wavelet transform, factor analysis and WE method

Exposure time	SG algorithm relative RMSE	FIR filtration relative RMSE	Wavelet transform relative RMSE	Factor analysis relative RMSE	WE method relative RMSE
1 s	1.47×10^{-1}	1.54×10^{-1}	1.45×10^{-1}	1.48×10^{-1}	8.21×10^{-2}
5 s	9.78×10^{-2}	9.94×10^{-2}	9.67×10^{-2}	1.10×10^{-1}	7.08×10^{-2}

The excellent performance of WE method can be attributed to two factors. One factor is that the synthesis of narrow-band measurements from low-SNR Raman spectra, in which Raman signals are integrated in the wavenumber dimension thus improving the SNR in narrow-band measurements. This works because shot noise dominates in typical Raman measurements [188, 189]. A similar strategy, i.e. integration of Raman signals over time or using a long exposure time in data acquisition, is used more often in practice [190]. The other factor is that WE method takes advantage of prior information about samples contained in Wiener matrix. Note that Wiener matrix is created in the calibration stage, in which Raman spectra with high SNR measured from similar samples are used and associated with narrow-band measurements. Unfortunately, the second factor is also responsible for the limitation of WE method, i.e. the Wiener matrix has to be derived from Raman spectra with high SNR measured from similar samples in the calibration stage prior to spectral recovery from low-SNR measurements. It would work better if the spectral variation across samples is smaller. For this reason, WE method would not work well if the calibration data set is very different from the test data set or when it is impossible to obtain the calibration data set. However, it should be pointed out that the calibration data set is often available in most biomedical applications. This fact has been utilized in many earlier researches in medical diagnostics [118, 119] thus not a problem in these applications.

Compared with other advanced methods, the advantages of our method include its simplicity, short processing time and excellent performance. The iterative three-point

zero-order SG filter method [191] is a fully automatic noise reduction method based on SG smoothing. Compared with this method, our method is simpler since it does not need iteration thus would be faster. In addition, our method always shows significant improvement in spectral shape preservation compared with SG filters based methods. For example, the iterative SG method always shows the peak height reduction problem while the method based on Wiener estimation shows much better performance on peak preservation in Fig. 7.3 and 7.4 of this chapter. Other advanced noise reduction methods based on regularization, e.g. Chi-squared based filters method [192] and matrix-based two dimensional regularization algorithm [193], are more complex and more time consuming than the proposed method due to the iterations required to satisfy the stop criterion. In contrast, our method requires a calibration data set, but the calibration data set is not needed in the test stage for data processing once the Wiener matrix is created, which not only takes advantage the prior information contained in the calibration data set but also accelerates data processing dramatically.

7.5 Conclusions

In this chapter, we develop a method based on spectral reconstruction to recover Raman spectra with low signal-to-noise ratio (SNR). Wiener estimation is used in this method to recover the high-SNR Raman spectra from narrow-band measurements that are synthesized from low-SNR Raman spectra. The synthesis of narrow-band measurements from low-SNR Raman spectra eliminates the effect of noise by integrating the Raman signal along the wavenumber dimension, which is followed by

spectral reconstruction based on Wiener estimation to recover the Raman spectrum with high spectral resolution. Non-negative principal components based filters are used in the synthesis to ensure that most variance contained in the original Raman measurements are retained. The method was evaluated on 25 Raman measurements from agar phantoms and 20 Raman measurements from bacteria samples. The agreement in peak locations between reference Raman spectra and Raman spectra recovered using WE (Wiener estimation) method was excellent and spectral shape information was mostly preserved. The relative mean root mean square errors (RMSEs) in cases of both with and without fluorescence background removal were small. In contrast, four commonly used de-noising methods, i.e. SG (Savitzky-Golay) method, FIR (finite impulse response) filtration, wavelet transform and factor analysis, showed significantly worse performance. Therefore, WE method represents a new alternative method for noise removal in those applications where short data acquisition yields Raman spectra with low SNR but creating a calibration data set is feasible.

Chapter 8: Minimization of Calibration Data Set in Spectral Reconstruction Based on Wiener Estimation

8.1 Introduction

In Chapter 6, we have successfully reconstructed Raman spectra from narrow-band measurements, which is an alternative way of Raman imaging. Raman data acquisition is generally slow due to inherently weak Raman signals, which prohibits Raman spectroscopic imaging from being used to investigate fast changing phenomena especially in biological samples[117]. By using the proposed method in Chapter 7, narrow-band Raman measurements can compensate for the weak Raman signal at each wavenumber by performing integration in the wavenumber dimension, in which the Raman spectrum with high spectral resolution can be reconstructed from the narrow-band measurements. The approach of narrow-band Raman measurements at each pixel followed by spectral reconstruction can facilitate the realization of fast Raman imaging. However, this method is limited in the requirement of a calibration data set, in which the calibration samples have to be similar to test samples in Raman features. Therefore, a new calibration data set is often needed for every type of samples, which implies a huge burden and prevents this Raman imaging approach from being widely adopted.

We propose a method to create a universal calibration dataset for Raman reconstruction to overcome this limitation. In our method, the calibration dataset is obtained using Raman spectra measured from basic biochemical components in

samples instead of actual samples. Because a general category of samples, such as human cells, share the same set of basic biochemical components, the calibration dataset based on these biochemical components is applicable to all samples in the same category and only a handful number of Raman measurements are needed to create such a universal calibration dataset. In this study, this approach was tested for phantoms with three basic biochemical components. A universal calibration dataset was created from 27 liquid phantoms in which three basic biochemical components were mixed. The Raman spectra reconstructed from the synthesized narrow-band measurements of phantom samples were compared to those measured from the same phantom samples. The results demonstrated the excellent performance of Raman reconstruction using the universal calibration dataset compared to the measured Raman spectra.

8.2 Materials and Methods

The phantoms were made by dissolving urea (V3171, Promega corporation, US), potassium formate (294454-500G, Sigma-Aldrich, US) and monosodium phosphate (20233-1KG, Affymetrix, US) in distilled water and mixing them. A total of 9 calibration phantoms were urea solution (0.5M, 1M and 1.5M), potassium formate solution (0.5M, 1M and 1.5M) and monosodium phosphate solution (0.75M, 1.5M and 2.25M). A total of 27 test phantoms were created by mixing urea (0.5M, 1M and 1.5M), potassium formate (0.5M, 1M and 1.5M) and monosodium phosphate (0.75M, 1.5M and 2.25M) in all possible combinations. The 9 calibration phantoms were used

as the calibration data set and the 27 test phantoms were used as the test data set in this study.

Raman spectra were measured over a range from 600 cm^{-1} to 1800 cm^{-1} , by using a micro-Raman system (innoRam-785S, B&W TEK, US) coupled to a video microscope sampling system (BAC151A, B&W TEK, US). The excitation wavelength was 785 nm and the spectral resolution was 4 cm^{-1} . The exposure time for Raman spectra was 10 s and each spectrum was accumulated for 30 times.

The narrow-band measurement c was simulated according to Eq. (8.1) when ignoring the noise term.

$$c = Fs \quad (8.1)$$

where s ($m \times 1$ matrix, in which m is the number of wavenumbers) is the Raman spectrum with fluorescence background and F ($n \times m$ matrix, in which n is the number of filters) represents the transmission spectra of the filters.

In Wiener estimation, a Wiener matrix W ($n \times m$ matrix) is used to transform narrow-band measurements c ($m \times 1$ matrix) into the corresponding Raman spectrum $\check{s}$ ($n \times 1$ matrix),

$$\check{s} = Wc \quad (8.2)$$

so that the mean square error between the original and estimated spectra is minimized.

The Wiener matrix W is given^[86] by Eq. (8.3) when ignoring the noise term.

$$W = K_s F^T (F K_s F^T)^{-1} \quad (8.3)$$

where

$$K_s = E[ss^T] \quad (8.4)$$

In Eq. (8.3) and (8.4), the superscript “T” represents matrix transpose, the superscript “ -1 ” represents matrix inverse and $E[\cdot]$ represents an ensemble average. Plugging Eq. (8.4) into Eq. (8.3) yields

$$W = E[sc^T][E[cc^T]]^{-1} \quad (8.5)$$

The PCs (principal components) based filter was used to generate the narrow-band measurements. The relative root mean square error (RMSE) of the reconstructed Raman spectrum after the removal of fluorescence background, relative to the measured Raman spectrum in which fluorescence background was also removed in the same manner, was computed as in Eq. (8.6).

$$\text{Relative RMSE} = \left[\frac{\sum_{i=1}^N [R_r(\lambda_i) - R_m(\lambda_i)]^2}{N \times \max[R_m(\lambda_i)]^2} \right]^{1/2} \quad (8.6)$$

where R_r and R_m are the reconstructed Raman spectrum and the measured Raman spectrum (both after fluorescence background removed), respectively, λ_i is the i -th wavenumber (i is varied from 1 to N) and the function, $\max[]$, returns the maximum intensity of the input spectrum.

8.3 Results

Figure 8.1 shows the Raman spectra of urea (0.5M, 1M and 1.5M), potassium formate (0.5M, 1M and 1.5M) and monosodium phosphate (0.75M, 1.5M and 2.25M) in the calibration dataset.

Table 8.1 shows the relative RMSE for different number of PCs based filters using the universal calibration dataset and the traditional calibration dataset. The number of the PCs based filters was varied from 2 to 7, because the reconstruction accuracy starts to drop when using 7 PCs for the universal calibration dataset. It can be seen that the relative RMSE reaches a minimum when using 6 PCs based filters for the universal

calibration dataset. Compared with the traditional calibration dataset, the reconstruction accuracy using the universal calibration dataset decreases by a range of percentages from 0.7% to 4.7%. All the results shown here were obtained after removing fluorescence background by fitting the fifth-order polynomial to the spectrum and subtracting the polynomial from it.

Fig. 8. 1 Raman spectra of (a) urea, (b) potassium formate and (c) monosodium phosphate in the calibration dataset.

Fig. 8. 2 (a) Best case, (b) typical case, (c) worst case of the reconstructed Raman spectra when using 6 non-negative PCs based filters, and (d) shows the transmittance spectra of 6 non-negative PCs based filters after normalization

Table 8. 1 Relative RMSE for different numbers of PCs based filters by using the universal calibration dataset and traditional calibration dataset

PC number	Relative RMSE for universal calibration dataset	Relative RMSE for traditional calibration dataset
2	0.0920	0.0449
3	0.0851	0.0259
4	0.0256	0.0063
5	0.0127	0.0060
6	0.0116	0.0035
7	0.0119	0.0030

Figure 8.2 shows the best, typical and worst cases of Raman reconstruction. The

relative RMSEs for the best, typical and worst case are 0.0061, 0.0109 and 0.0174, respectively. From Table 8.1 and Fig. 8.2, we can find that both the peak locations and peak intensities match very well between the estimated Raman spectra and original Raman spectra. Therefore, the universal calibration dataset can be used to reconstruct Raman spectra with excellent accuracy.

8.4 Conclusions

In this chapter, we proposed a method of creating a universal calibration dataset by measuring basic biochemical components and tested the method in liquid phantoms with three basic biochemical components. The results demonstrated the excellent performance of Raman reconstruction using the universal calibration dataset compared to that using the traditional calibration dataset and the measured Raman spectra. We are further developing this method for biological samples.

Chapter 9: Conclusions and Future Directions

9.1 Conclusions

This dissertation presents a series of studies on the development of fast spectral imaging technique for diffuse reflectance, fluorescence and Raman measurements in biomedical applications. In these studies, two novel reconstruction algorithms, including modified Wiener estimation and sequential weighted Wiener estimation, was developed. The methods were tested in studies involving simulations, several phantom studies and one animal study. The proposed methods showed the enormous potential of realizing fast spectroscopic imaging to observe fast changing phenomena in biomedical applications. The main findings of these studies are summarized as follows.

First, full optical spectra can be reconstructed from a few narrow-band/wide-band measurements and advanced reconstruction methods could improve the reconstruction accuracy and speed significantly. The development of modified Wiener estimation in Chapter 3 showed significant improvement in the reconstruction accuracy of estimated diffuse reflectance spectra from RGB color values compared to the traditional Wiener estimation. Moreover, the method of sequential weighted Wiener estimation in Chapter 4 could improve the estimation of key tissue parameter directly from the RGB values and their ratios. This method is fast thus it may allow real time monitoring of key tissue parameters in a large tissue area when combined with diffuse reflectance imaging. The animal study in Chapter 5 further validated the potential of using narrow-band/wide-band measurements and their ratios in clinical diagnosis.

Second, the proposed spectroscopic imaging technique can be extended to Raman spectroscopy. In Chapter 6, it was found that both Raman spectra with and without fluorescence background could be reconstructed from narrow-band measurements based on the proposed spectroscopic imaging technique. Higher signal to noise ratio and lower fluorescence background could improve the reconstruction accuracy, thus various fluorescence suppression methods, e.g. shifted excitation Raman difference spectroscopy, Fourier transformed Raman spectroscopy, and temporal gating, would further improve the reconstruction accuracy in this method. In addition, the proposed method has a significant advantage when measuring Raman spectra with extremely low SNR as shown in Chapter 7. The synthesis of narrow-band measurements from low-SNR Raman measurements reduce the influence of noise by integrating the Raman signal along the wavenumber dimension, which is followed by spectral reconstruction based on Wiener estimation to recover the Raman spectra with a high spectral resolution. Therefore the WE method is a new alternative way for noise removal in those applications where short data acquisition yields Raman spectra with low SNR but creating a calibration data set is feasible.

Finally, the major limitation of the proposed spectroscopic imaging techniques, i.e. the requirement of a new set of calibration data for a different type of samples, was addressed in Chapter 8. It was found that a universal calibration dataset can be created by measuring basic biochemical components in the samples instead of actual samples. Because common biomedical samples, such as human cells, share the same set of basic biochemical components, the calibration dataset based on these biochemical

components is applicable to all samples and only a handful number of Raman measurements are needed to create such a universal calibration dataset. Therefore, the proposed spectroscopic imaging techniques can be adopted for common biomedical samples with only a small number of calibration data measurements required.

9.2 Future Direction

In this series of studies, we have mainly focused on the spectroscopic imaging techniques and the advanced algorithms for spectral reconstruction. Further work is needed to simplify the system, improve the reconstruction accuracy, shorten the computation time and simplify the acquisition of calibration data set. The major future directions are described as follows.

A. The modified WE in Chapter 3 only evaluated the outcome of synthesizing three additional narrow-band measurements and the effect of using a different number of synthetic filters was not investigated. More synthetic measurements usually would improve the accuracy of Wiener estimation. However, this trend could be counteracted by the addition of noises and uncertainty in these synthetic measurements at certain point. Therefore, further work needs to be done to optimize the number of synthetic filters and refine the modified WE to extend the applicability and improve the accuracy.

B. The sequential weighted WE and the proposed optical system in Chapter 4 was designed for homogenous tissue phantoms. However, human tissues, such as the skin tissue, are usually heterogeneous in nature and the optical properties are different

among tissue layers. Therefore, the calibration data may need to be obtained from a more realistic tissue phantom model in clinical applications. For example, a series of two-layered skin phantoms including melanin in the epidermis with a range of epidermal thicknesses and optical properties in both layers will yield a better set of calibration data for clinical measurements. However, such a multi-layered phantom will contain much more parameters, thus a depth sensitive color imaging system and an advanced estimation method will need to be developed to selectively acquire color images from each layer to improve the accuracy of estimated parameters. Another issue is that the calibration data set and test data set in Chapter 4 are device-dependent. This is inconvenient in biomedical applications because the estimation accuracy may degrade when there is any change in environmental light or system specifications. Further work will need to be done to convert all the calibration data and test data into the CIE XYZ color space, which is a device-independent color space. The computing time for sequential weighted Wiener estimation is also relatively long, which is around 50 times of that for the traditional Wiener estimation. The problem could be solved by the prior calculation of the weighted Wiener matrix, which can be stored in a database according to the corresponding weights. When certain weights appear, the Wiener matrix can be directly retrieved from the database, which can save the computing time dramatically.

C. Although the diffuse reflectance and fluorescence imaging system showed the feasibility of early prediction of occlusion in Chapter 5, there are several issues to be addressed in clinical applications. The currently large system needs to be made

portable first. Moreover, a method for ambient light suppression is needed so that the proposed imaging system is able to work under normal lighting condition for clinical use. Furthermore, a mechanism needs to be established to remove the need of manual focusing and positioning for convenient use by a clinician.

D. An online database including the calibrated Raman spectra of basic biochemical components and standard narrow-band measurements would save repeated measurements for creating the universal calibration dataset in spectroscopic Raman imaging. The standard narrow-band measurements should be device-independent, which can be converted from the other user's narrow-band measurements using a method similar to that shown in Chapter 4.

E. A Raman imaging system will be built to collect both spatial and spectral information simultaneously. In this method, a method to generate a filter with an arbitrary transmission spectrum will be valuable to realize the transmission of PCs based filters and to reduce the cost. A method to suppress fluorescence background could be induced into the Raman imaging system, which will improve the reconstruction accuracy as shown earlier..

In summary, the proposed spectroscopic imaging technique is promising in achieving fast spectroscopic imaging and being adopted clinically. However, this method is far from being complete. Several challenges need to be addressed as shown above, which also represent numerous opportunities for new technical development. With the advances in all these aspects in the future, this project is expected to generate a rapid and cost effective optical spectroscopic imaging system for both clinical applications

and biological science research.

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Academic Journals

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3. C. Zhu, **S. Chen**, C. H. Chui and Q. Liu, Early prediction of skin viability using visible diffuse reflectance spectroscopy and autofluorescence spectroscopy, *Plastic and Reconstructive Surgery*, 134(2), 240e-247e, (2014)
4. **S. Chen**, Y. H. Ong, and Q. Liu, Fast reconstruction of Raman spectra from narrow-band measurements based on Wiener estimation, *Journal of Raman Spectroscopy*, 44(6), 875-881 (2013)
5. **S. Chen**, and Q. Liu, Modified Wiener estimation of diffuse reflectance spectra from RGB values by the synthesis of new colors for tissue measurements, *Journal of Biomedical Optics*, 17(3), 030501 (2012)

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1. **S. Chen**, X. Lin, C. Yuen, S. Padmanabhan, R. Beuerman, Q. Liu, Noise removal of Raman spectra with extremely low signal to noise ratio, *Proc. SPIE 9318, Optical Biopsy XIII* (February 11, 2015)
2. C. Zhu, **S. Chen**, C. H. Chui, B. Tan, Q. Liu, Assessment of venous and arterial

occlusion in skin flaps using visible diffuse reflectance spectroscopy and autofluorescence spectroscopy in a rodent model, Proc. SPIE 9303, Photonics in Dermatology and Plastic Surgery (February 7, 2015)

3. **S. Chen**, X. Lin, Q. Liu, Fast reconstruction of Raman spectra from wide-band measurements of Raman signals with fluorescence background. Proc. SPIE 8940, Optical Biopsy XII (February 4, 2014)

4. **S. Chen**, X. Lin, C. Zhu, Q. Liu, Rapid estimation of key tissue parameters from wide-band diffuse reflectance measurements based on sequential weighted Wiener estimation. Proc. SPIE 8935, Advanced Biomedical and Clinical Diagnostic and Surgical Guidance Systems XII (February 2, 2014)

5. C. Zhu, **S. Chen**, C. H. Chui, B. Tan, Q. Liu, Early prediction of skin flap viability using visible diffuse reflectance spectroscopy and autofluorescence spectroscopy. Proc. SPIE 8935, Advanced Biomedical and Clinical Diagnostic and Surgical Guidance Systems XII (February 2, 2014)

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