MUELLER-MATRIX-BASED ANALYSIS FOR SECOND-HARMONIC GENERATION IMAGING

BY

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THESIS

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ABSTRACT

Second-harmonic generation microscopy takes advantage of second-order non-linear property for imaging of biological structures. Invoking quantitative image analysis techniques facilitates the assessment of tissues in varying states.

This thesis presents a quantitative description of the relative structural difference in collagen-based tissues by second harmonic generation (SHG) imaging techniques involving polarization investigation. The Mueller matrix formalism demonstrates a polarization-based analysis approach for assessment of varying samples.

The associated second-order Mueller matrices are generated by applying image processing algorithms to sub-grids of image sets obtained from a forward imaging microscope. By defining scalar metrics such as the degree of polarization and the depolarization, a quantitative evaluation of the samples under investigation is provided. A discussion of the capabilities for tissue assessment and techniques for improvements in the data acquisition speed and analysis will also be proffered.
To family and friends who have supported me through my endeavors.
ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

1.1 Overview

Nonlinear imaging techniques in biomedical imaging provide many advantages in assessing and characterizing sample properties. By exploiting nonlinear effects in biological samples, higher-contrast, higher-resolution images than in linear imaging can be obtained. Examples of nonlinear imaging modalities are multiphoton fluorescence (two- and three-photon), harmonic generation (second- and third-harmonic) and the coherent Raman scattering (Stokes and anti-Stokes).

The second harmonic generation (SHG) imaging technique is a widely used nonlinear optical technique. It is based on the second harmonic generation effect, in which photons at a certain frequency interact in a material to yield photons at twice the frequency (or half the wavelength) of the original. The SHG process was first demonstrated by Franken et al. in 1961, facilitated by the invention of the laser (or optical maser as it was referred to) [1]. This discovery opened up new application frontiers such as frequency doubling of laser using birefringent crystals [2] and spectroscopic investigation of processes at interfaces [3].

One of the earliest demonstration of SHG imaging on biological samples was reported by Freund et al. [4] in 1986. Using a scanning second-harmonic microscopy setup, the existence of a discrete network of structures and polar surfaces on a sample of rat-tail tendon was revealed. SHG microscopy has subsequently found varied implementations in biological imaging. By applying this technique to photoreceptor cells, its capability for imaging living cells was shown [5]. It has also been used for visualizing biomolecular arrays in cells and tissues [6]. An advantage of SHG imaging lies in its generation from induced polarization scattering as opposed to absorption. This reduces
the photobleaching and phototoxicity associated with fluorescent methods. Another benefit is its sensitivity to non-centrosymmetric structures (that is, those lacking a center of inversion symmetry). An example of a biological tissue having the property of non-centrosymmetry is fibrillar collagen.

Further advances in SHG imaging have been achieved by applying quantitative analysis techniques. This has enabled extensive assessment of tissue samples in varying states. Metrics such as fiber orientation anisotropy and structural organization have been used for the investigation of rat cervical tissue [7], collagen fiber organization from biological tissues [8], discrimination of horse tendon based on injury [9] and studies of changes in porcine bone due to ageing [10]. Other quantitative measures used include the second-order nonlinear susceptibility tensor elements for description of collagen fiber in breast biopsies [11].

By further exploring the polarization SHG properties of samples, the second-order Mueller matrix analysis for assessment of tissues and the extraction of associated scalar metrics is demonstrated. This process involves the acquisition of images with known input polarization states, and using image analysis algorithms to extract the relevant parameters for the sample under investigation.

1.2 Organization of Chapters

Chapter 2 explores the motivation and background for quantitative second-harmonic generation techniques. Chapter 3 lays the mathematical framework for second-order Mueller matrix SHG analysis and derives metrics to be used. Chapter 4 describes the experimental setup for imaging, and highlights results with discussion. Chapter 5 provides a summary of the thesis. The appendix includes a summary of relevant parameters.
2.1 Motivation

Collagen is the most prevalent protein in the human body, forming a major component of skin, cornea and bone tissues [12]. Its structure is triple-helical consisting of α-chains of molecules bonded to each other. The molecules are assembled into fibrils of diameters 20–250 µm, and then further aggregated into fibers with diameters of up to 500 µm [13]. There exists up to 28 different types of collagen, with Type 1 being the most abundant.

Collagen is found in the extracellular matrix (ECM) surrounding cells, and plays an active role in cell function and development. For example, change in the organization of collagen is shown to be important in the degenerative eye disorder, keratoconous [14]. This occurs when the cornea is unable to preserve its shape against intraocular pressure of the eye fluids due to weakening of the collagen fibrils, which provide support and rigidity. Hence, the cornea takes a distorted shape, leading to distorted vision. Another illustration of the importance of collagen is shown in its active participation in the tumor metastatic process [15]. Its degradation products during multiple steps of metastasis aid in promoting the spread of a tumor beyond its initial site.

Hence, imaging collagen while highlighting its organization and composition will enable a deeper understanding of the effect of fibrillar collagen structure on function, and facilitate development of therapies for conditions involving structural abnormalities. Desired requirements for an imaging system include high specificity to collagen and minimum specimen damage to the biological sample. Other abilities such as three-dimensional sectioning and quantification are advantageous for robust description of samples. One technique used for imaging is polarized light microscopy (PLM), which takes advantage of the optical anisotropy properties of collagen [16]. However, its
restriction to two-dimensional imaging and non-specificity to collagen makes it a less preferred option. The scanning electron microscope has also been used for imaging collagen [17], and though it has very good resolution, it is a disruptive technique that can strongly alter the biology of the tissue due to metal deposition.

SHG microscopy provides a suitable method for imaging collagen. It demonstrates specificity to the non-centrosymmetric structure of collagen fibers, and can image it in isolation from other surrounding biological components. It has deeper penetration depth, since typical wavelengths used fall in the biological window (700-1300 nm), and provides no deposition of energy into the material since it is a coherent process. By reason of its intrinsic confocality within the thin focal volume where SHG occurs for focusing systems, optical sectioning is achievable.

2.2 Second-Harmonic Generation

Second-harmonic generation is the underlying phenomenon for the applied imaging technique. It is a nonlinear optical process in which incident light of a particular wavelength interacts in a medium having a non-centrosymmetric structure, to yield light at half the wavelength. The nonlinearity is negligible at low intensities, but becomes more appreciable at higher intensities, of the order of $10^5 - 10^8$ V/m [18].

The induced polarization density ($P$) due to incident field $E$ on a material can be expressed by

$$P = \chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + ...$$  \hspace{1cm} (2.1)

where $\chi^{(n)}$ is the $n$th-order susceptibility. By considering only the second term for SHG, the second-order induced polarization density is given as

$$P_l(2\omega) = \sum_{mn} \chi_{lmn} E_m(\omega) E_n(\omega) \quad l, m, n = 1, 2, 3$$  \hspace{1cm} (2.2)

where $\omega$ is the angular frequency, $\chi_{lmn}$ is a third rank tensor and $P(2\omega)$ is the component polarization density along the principal axes of the material. By taking advantage of symmetry conditions, Equation 2.2 can be simplified, and
the emitted SHG intensity then evaluated from the polarization components [19].

2.3 Quantitative Second-Harmonic Generation

Several techniques have been utilized in order to develop quantifiable parameters describing collagen structural organization and anisotropy properties from its SHG emission properties. An example is the forward to backward intensity ratio (F/B) as a function of excitation depth which has been used to highlight differences in morphology in different tissues [20–22].

Another quantitative approach is the Fourier-Transform Second-Harmonic Generation (FT-SHG) method. This approach has been employed to determine the preferred orientation and maximum spatial frequency of collagen fibers as a metric to investigate structural changes due to physical injury or disease [23]. It has also been combined with F/B ratio for a more rigorous description [9]. It was observed that injured tendon displayed less ordered fibers and larger F/B ratio distribution in comparison with normal tendon. In another study, FT-SHG was applied to investigate changes in fiber organization of bone as a function of age [24]. By exploiting the advantage of SHG for sectioning, FT-SHG was generalized for three-dimensional analysis [8] and used in studies of rat cervix [7].

2.3.1 Polarization-Resolved Second-Harmonic Generation

The optical anisotropic properties of collagen fibers have also been investigated by polarization analysis in the Polarization-Resolved Second-Harmonic Generation (PR-SHG) approach. By invoking symmetry conditions on the nonlinear susceptibility matrices, the SHG intensity can be modeled as a function of the polarization angle. Parameters such as the $d$-matrix ($d_{lmn}^{(2)} = \frac{1}{2}\chi_{lmn}^{(2)}$) are then defined, and $d$-values and $d$-ratios can be obtained from selected elements of the tensor $d$-matrix. A study on frozen sections of osteosarcoma, breast carcinoma and melanoma tumor tissues in comparison with normal tissues was done [25], with the collagen structure characterized by a specific $d$-value ($d_{22}$). Differences in structure was reflected in higher $d_{22}$ coefficients. A more detailed explanation of the $d$-ratio analysis is given
in Section 2.3.1.1.

2.3.1.1  $d$-Ratio Analysis for Polarization Investigation

PR-SHG analysis was conducted on breast tissue microarrays as a means for quantifying collagen structure for different pathological conditions by Ambekar et al. [11]. Following from Equation 2.2, and assuming the intrinsic permutation and Kleinmann symmetry conditions, the $d$ tensor can be written in contracted notation, and the induced polarization density at the second-harmonic for rectangular coordinates becomes:

\[
\begin{pmatrix}
P_x(2\omega) \\
P_y(2\omega) \\
P_z(2\omega)
\end{pmatrix} = 2
\begin{pmatrix}
d_{11} & d_{12} & d_{13} & d_{14} & d_{15} & d_{16} \\
d_{21} & d_{22} & d_{23} & d_{24} & d_{25} & d_{26} \\
d_{31} & d_{32} & d_{33} & d_{34} & d_{35} & d_{36}
\end{pmatrix}
\begin{pmatrix}
E_x(\omega)^2 \\
E_y(\omega)^2 \\
E_z(\omega)^2 \\
2E_x(\omega)E_y(\omega) \\
2E_x(\omega)E_z(\omega) \\
2E_y(\omega)E_x(\omega)
\end{pmatrix}
\] (2.3)

Using the more general $3m$ crystal class symmetry model as opposed to the simpler $C6$ model, the $d$-matrix becomes:

\[
\begin{pmatrix}
0 & 0 & 0 & 0 & d_{15} & d_{22} \\
-d_{22} & d_{22} & 0 & d_{15} & 0 & 0 \\
d_{31} & d_{31} & d_{33} & 0 & 0 & 0
\end{pmatrix}
\] (2.4)

where $d_{15}$, $d_{22}$, $d_{31}$, and $d_{33}$, are the only non-zero elements. Hence, the components of the polarization density can be expanded thus:

\[
\begin{align*}
P_x(2\omega) &= 2d_{15}E_x(\omega)E_z(\omega) - 2d_{22}E_x(\omega)E_y(\omega) \\
P_y(2\omega) &= d_{22}(-E_x^2(\omega) + E_y^2(\omega)) + 2d_{15}E_y(\omega)E_z(\omega) \\
P_z(2\omega) &= d_{31}(E_x^2(\omega) + E_y^2(\omega)) + d_{33}E_z^2(\omega)
\end{align*}
\] (2.5)

Suppose a collagen fiber under investigation is oriented as illustrated in Figure 2.1, with the E-field normal to the $y$-z plane, and $\alpha$ the angle between incident polarization and the z-axis. For relatively weak focusing of the incident beam, the longitudinal field component is negligible compared to
From Equation 2.6, the values of the tensor coefficients for fibers can be obtained by fitting the experimental plot of SHG intensity as a function of the linear input polarization modulation. The assessment technique proceeds with normalizing by $d_{31}$ and performing statistical comparison tests of the ratios using repeated measures ANOVA with between-subject factor for different breast pathologies.

2.3.1.2 Mueller Matrix-Based Analysis for Polarization Investigation

It is expected that the aggregate optical anisotropic properties of collagen fiber can be used as an assessment of structural or organizational alteration brought about by disease, injury or abnormality. Hence, the polarization-based analysis techniques present a powerful investigation tool for assessing tissue characteristics. Mueller matrix-based analysis for second-harmonic generation (MM-SHG) is another polarization investigation method that utilizes the Mueller matrix representation of systems by relating the output and
input light Stokes vector representations. This comprehensive approach can be extended to the extraction of vector and scalar metrics that can be studied for the assessment of tissue states.
3.1 Motivation

Characterizing the polarization interaction of optical elements can be approached using either the Jones or the Mueller calculus approach. While the former assumes a coherent addition of waves, the latter assumes an incoherent addition of waves [26]. The Jones analysis method is a mathematically rigorous technique, but has a shortcoming in being unable to describe unpolarized or partially polarized light. The Mueller analysis development is heuristic and has the benefit of dealing with measurable intensity values (the Stokes vectors) rather than amplitudes and phase values (Jones vectors).

Mueller matrix imaging polarimetry has been developed to measure the spatially dependent polarization properties of optical samples and systems, and extended to biological systems. Smith et al. utilized the technique for characterizing various dermatological diseases [27]. They showed in their studies that malignant moles were less depolarizing than surrounding tissues, while lupus lesions had rapidly varying retardance orientation. Jiao et al. [28] incorporated Mueller matrix analysis into an optical coherence tomography imaging method to investigate birefringence, diattenuation and intensity contrast for rat skin samples.

One of the postulates for the Mueller analysis approach involves assuming a linear relation between the input and output Stokes vectors. Hence, the question has been asked on whether a similar formalism can be extended to nonlinear light scattering [29]. A nonlinear Mueller model capable of relating input to output should prove very useful in describing the optical nonlinear polarization response of biological systems as an assessment tool.
3.2 Background

The linear Stokes vector describes the polarization state of light in a $4 \times 1$ vector form [30], and can be expressed in terms of combinations of measured intensity values at different chosen polarization bases.

\[
S = \begin{pmatrix}
  s_0 \\
  s_1 \\
  s_2 \\
  s_3
\end{pmatrix} = \begin{pmatrix}
  I \\
  pI\cos(2\chi)\cos(2\psi) \\
  pI\cos(2\chi)\sin(2\psi) \\
  pI\sin(2\chi)
\end{pmatrix} = \begin{pmatrix}
  I_H + I_V \\
  I_H - I_V \\
  I_P - I_P^* \\
  I_R - I_R^*
\end{pmatrix}
\] (3.1)

In Equation 3.1, $I$ stands for the total intensity, $p$ represents the degree of polarization, while $\psi$ and $\chi$ represent the orientation and ellipticity respectively. $H, V, P, P^*, R$ and $R^*$ stand for the 0°, 90°, 45°, −45°, right circularly polarized and left circularly polarized light, respectively.

The 1-photon linear Mueller matrix relationship between input ($s_\beta$) and output ($s_\alpha$) Stokes vectors can be described by

\[
s_\alpha = M_{\alpha\beta}^{(1)} s_\beta \quad \alpha, \beta = 0, 1, 2, 3
\] (3.2)

where $M_{\alpha\beta}$ is a $4 \times 4$ Mueller matrix describing the optical transformation of polarization state from an input Stokes vector to an output Stokes vector and the zero index is used for consistency with Stokes convention.

Extending to a multi-photon effect, the Mueller becomes an $(n+1)$ dimensional array where $n$ is the order of the effect. At the second harmonic, Equation 3.2 becomes

\[
s_\alpha = M_{\alpha\beta\gamma}^{(2)} s_\beta s_\gamma \quad \alpha, \beta, \gamma = 0, 1, 2, 3
\] (3.3)

where the $M_{\alpha\beta\gamma}$ is a $4 \times 4 \times 4$ second-order Mueller array relating two input Stokes vector to yield an output Stokes vector.

For identical input such as in second-harmonic generation, we can reduce the three-dimensional $4 \times 4 \times 4$ array acting on two $4 \times 1$ input vectors to a $4 \times 9$ matrix acting on one $9 \times 1$ input vector so that $M_{\alpha\beta\gamma}^{(2)} \Rightarrow M_{\alpha\Gamma}^{(2)}$ and

\[
s_\alpha = M_{\alpha\Gamma}^{(2)} s_\Gamma \quad \alpha = 0, 1, 2, 3 \quad \Gamma = 0, 1, 2, ..., 8
\] (3.4)

A non-rigorous argument for reduction of parameters follows. The intensity
for a two-photon process in terms of amplitude following from Equation 2.2 is given by

\[ A_l = \sum_{m,n=H,V} \chi_{lmn} E_mE_n \equiv 4 \text{ terms} \quad (3.5) \]

\[ I_l = A_l A_l^* \equiv 16 \text{ terms} \quad (3.6) \]

If the two photons are identical, it follows that permutation symmetry holds, and \( \chi_{lmn} = \chi_{lnm} \).

\[ A_l = \chi_{lHH} E_H E_H + \chi_{lVV} E_V E_V + 2\chi_{lHV} E_H E_V \quad \equiv 3 \text{ terms} \quad (3.7) \]

\[ I_l = A_l A_l^* \equiv 9 \text{ terms} \quad (3.8) \]

Hence, for the two-photon process in a single beam, sixteen (4×4) parameters can be reduced to nine (9×1) parameters, the so-called double Stokes vector [31]. The expression for the double Stokes vector from the linear Stokes vector can be obtained starting from the second-order polarization [32].

\[ P_l = \sum_{mn} \chi_{lmn} E_mE_n \quad m, n = 1, 2 \quad (3.9) \]

\[ = \sum_A \chi_{lA} \Psi_A \quad A = 1, 2, 3 \quad (3.10) \]

By writing the state function as \( \Psi^T = \begin{pmatrix} E_H^2 & E_V^2 & 2E_H E_V \end{pmatrix} \), the coherency matrix can be written as a dyadic product of the state function and its conjugate leading to

\[ \rho = \Psi \cdot \Psi^* = \begin{pmatrix} E_H^2 & E_H^* E_V & 2E_H E_V^* \\ E_V^2 & E_V^* E_H & 2E_V E_H^* \\ 2E_H E_V & 2E_V E_H & 4E_H E_V^* \end{pmatrix} \quad (3.11) \]

By using the relation \( S_N = Tr(\rho \lambda_N) \) where \( \lambda_N \) are the Gell-Mann matrices (Equation A.1), the expression for the double Stokes vectors can thus be
obtained [32]:

\[
S_D = \begin{pmatrix}
S_1 \\
S_2 \\
S_3 \\
S_4 \\
S_5 \\
S_6 \\
S_7 \\
S_8 \\
S_9
\end{pmatrix} = \begin{pmatrix}
\sqrt{\frac{1}{6}}(3s_6^2 - s_1^2) \\
\frac{1}{12}(5s_2^2 - 3s_0^2) \\
-s_0s_1 \\
\frac{1}{2}(s_2^2 - s_3^2) \\
s_2(s_1 + s_0) \\
-s_2(s_1 - s_0) \\
-s_2s_3 \\
s_3(s_1 + s_0) \\
s_3(s_1 - s_0)
\end{pmatrix}
\]

where \( E_H E_H^* = \frac{1}{2}(s_0 - s_1) \), \( E_V E_V^* = \frac{1}{2}(s_0 + s_1) \), \( E_H E_V^* = \frac{1}{2}(s_2 + is_3) \), \( E_V E_H^* = \frac{1}{2}(s_2 - is_3) \).

To obtain the output in conventional \( 4 \times 1 \) vector formalism, there is need for the second-order Mueller matrix to be expressed in a \( 4 \times 9 \) matrix form. This can be obtained by generating and solving a system of equations with pre-determined inputs and measured nonlinear output.

### 3.3 Mueller Matrix Analysis

A series of nine known input PSG states are generated. The states are chosen such that they present a symmetric disposition on the Poincaré sphere (a slightly different model from [31]). The polarization states are chosen as shown in Figure 3.1, and the Stokes and double Stokes vectors described later in Table B.3 of the appendix.

For each input state, six PSA images should be acquired. However, it is usually enough to obtain four PSA images \((H, V, P, R)\) since the other two can be derived from these four \((P^* = H + V - P, R^* = H + V - R)\). The set of nine equations to solve are:

\[
M_D \cdot H^{(i)} = H^{(o)}, \quad M_D \cdot V^{(i)} = V^{(o)}, \quad M_D \cdot P^{(i)} = P^{(o)} \\
M_D \cdot P^{(i)} = P^{(o)}, \quad M_D \cdot R^{(i)} = R^{(o)}, \quad M_D \cdot R^{*(i)} = R^{*(o)} \\
M_D \cdot H_P^{(i)} = H_P^{(o)}, \quad M_D \cdot V_{R^*}^{(i)} = V_{R^*}^{(o)}, \quad M_D \cdot P_{R^*}^{(i)} = P_{R^*}^{(o)}
\]

\[(3.13)\]
where the \((i)\) and \((o)\) superscripts stand for input and output respectively and \(M_D\) is the double Mueller matrix. The input Stokes state is the \(9 \times 1\) vector representation, while the output state is the \(4 \times 1\) representation which involves the degree of polarization term \(p\) and expressed as

\[
A^{(o)} = \begin{pmatrix}
  AH + AV \\
  AH - AV \\
  AP - AP^* \\
  AR - AR^*
\end{pmatrix}
A \equiv H, V, P, P^*, R, R^*, H_P, V_{R^*}, P_{R^*}^* \quad (3.14)
\]

Therefore, for each region to be assessed, a total of 54 images will be enough to extract the \(4 \times 9\) Mueller matrix. The individual input and output matrices obtained can be concatenated to obtain consolidated \(9 \times 9\) input and \(4 \times 9\) output matrices respectively.

\[
U^{(i)} = \begin{pmatrix}
  H^{(i)} & V^{(i)} & P^{(i)} & P^{(i)*} & R^{(i)} & R^{(i)*} & H_P^{(i)} & V_{R^*}^{(i)} & P_{R^*}^{(i)}
\end{pmatrix} \quad (3.15)
\]

\[
U^{(o)} = \begin{pmatrix}
  H^{(o)} & V^{(o)} & P^{(o)} & P^{(o)*} & R^{(o)} & R^{(o)*} & H_P^{(o)} & V_{R^*}^{(o)} & P_{R^*}^{(o)}
\end{pmatrix} \quad (3.16)
\]

A \(9 \times 1\) row vector degree of polarization metric can be deduced from \(U^{(o)}\)
by invoking the relation on each column $m$

$$p_m = \sqrt{U_{m1}^{(o)2} + U_{m2}^{(o)2} + U_{m3}^{(o)2}} \over U_{m0}$$  \hspace{1cm} (3.17)

Subsequently, a scalar degree of polarization metric $p$ and a depolarization term $\Delta$ can be obtained via:

$$p = \frac{1}{9} \sum_{m=1}^{9} p_m \hspace{1cm} \Delta = 1 - p \hspace{1cm} (3.18)$$

This metric is a measure of the property of aggregate degree of polarization.

In principle, for fully polarized input, the output Stokes vector is depolarized by a certain amount, and this is obtained for the different input orientations. For example, a horizontally polarized light input yields an output SHG Stokes vector, with a certain degree of polarization obtained after measurement. The degree of polarization terms is obtained across the concatenated output to form a row vector, and then an aggregate scalar. By inference, aggregate depolarization in the medium can be gotten from the degree of polarization by Equation 3.18.

Eventually, the double Mueller matrix can be determined by solving

$$M_D = U^{(o)} \cdot [U^{(i)}]^{-1}$$ \hspace{1cm} (3.19)
CHAPTER 4

ANALYSIS APPLICATION AND RESULTS

4.1 Experimental Setup

Figure 4.1 shows the setup used for experiments. The SHG signals are collected in the forward direction in order to eliminate the use of dichroics which have inferior polarization preserving properties when compared with metal mirrors. The laser source used was a Ti:Sapphire laser (Spectra-Physics Mai-Tai HP DeepSee) producing 100 fs pulses at a repetition rate of 80 MHz with an excitation wavelength centered spectrally at 780 nm. Galvanometer-based scanning mirrors (Thorlabs GVS012) are used to sweep the beam over a rectangular field of view at the sample plane. A 4-f system consisting of two lenses (L1 and L2) is used to translate the galvanometer plane to the back aperture of the condenser.

The setup contains a polarization state generator (PSG) system at the input and a polarization state analyzer (PSA) system at the output. The PSG consists of a linear polarizer and waveplate combination (half waveplate, quarter waveplate or both) for generating desired polarization states. Ideally, the same number of optical components should be used for the polarization generation to ensure near constant power delivery to the sample. However, transmittance of the waveplates were measured to be greater than 93%, and so the effect of an extra optical component could be ignored. The PSA contains a quarter waveplate and linear polarizer combination. The angle combinations for the optical components are shown in Tables B.1 and B.2 of the appendix.

The condenser and objective lenses were chosen based on numerical aperture (NA) as a compromise between resolution (increases with higher NA) and polarization preservation at focus (decreases with higher NA). For the current study, a condenser with 0.65 NA (Olympus 40× PLAN N) and ob-
jective with 0.8 NA (Olympus 50× MPlan FL N) were used for imaging. A tube lens used for focusing, a laser-blocking short-pass filter (Semrock FF01-680/SP-25) for illumination rejection and an SHG band-pass filter (Semrock FF01-390/BP-18-25) for narrow band filtering make up part of the optical setup. An electron multiplying charge-coupled device (Hamamatsu EMCCD C9100-13) camera is used as the detector with a gain of 200× and an exposure time of 1 s.

Figure 4.1: Experimental setup.

4.2 Results and Discussion

The samples were prepared by cutting into thin parts, placing in a mold and pouring optimal cutting temperature (OCT) compound on it. The molds were then placed in a box filled with dry ice, so that the OCT freezes the sample. The samples are then cut, and mounted on a microscope slide. Separate samples were also paraffin-embedded by cutting, fixing in formalin and mounted in molds. Three samples (pig tendon samples with thicknesses 25 μm and 5 μm, and pig skin sample with thickness 5 μm) were used in the study.
For each sample, three separate regions were imaged over a field-of-view of about 70 µm. The set of 54 images corresponding to PSA-PSG state combinations was obtained. A reference image for each sample was divided into cells using a grid framework. The output concatenated Stokes vector and second-order Mueller matrix representations per grid were obtained using an algorithm which incorporates Equations 3.14 to 3.19. Furthermore, the depolarization scalar metric for the sample was obtained.

Figure 4.2 shows the histogram plot of the average depolarization across the grid cells of three regions each for pig tendon and pig skin at 5 µm. This was investigated as a study of parameter variation across different samples having the same thickness.

![Figure 4.2](image)

**Figure 4.2**: Histogram plot of average depolarization across different samples.

The effect of grid size on the polarization parameters was studied and the results are shown in Figure 4.3. For various grid numbers (16 × 16, 32 × 32, 64 × 64 and 320 × 320, the latter corresponding to the grid cell size of one pixel), the histogram distribution of average depolarization was plotted, with the mean and standard deviation observed. The distributions for all the grid numbers used were similar. Above 32 × 32, difference in parameter distribution was largely unnoticeable.
<table>
<thead>
<tr>
<th>Grid Size</th>
<th>Pig Tendon 5 µm thickness</th>
<th>Pig Skin 5 µm thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 × 16</td>
<td>Mean = 0.765 Std = 0.049</td>
<td>Mean = 0.866 Std = 0.033</td>
</tr>
<tr>
<td>32 × 32</td>
<td>Mean = 0.765 Std = 0.052</td>
<td>Mean = 0.866 Std = 0.039</td>
</tr>
<tr>
<td>64 × 64</td>
<td>Mean = 0.765 Std = 0.053</td>
<td>Mean = 0.866 Std = 0.042</td>
</tr>
<tr>
<td>320 × 320</td>
<td>Mean = 0.761 Std = 0.054</td>
<td>Mean = 0.861 Std = 0.044</td>
</tr>
</tbody>
</table>

Figure 4.3: Histogram plot of average depolarization for samples with varying grid sizes during algorithm analysis.
The analysis method was further applied to samples of different thicknesses as shown in Figure 4.4. Different regions for the pig tendon samples with thicknesses of 5 \(\mu\text{m}\) and 25 \(\mu\text{m}\) were studied, and the average depolarization histogram plot for the three regions was obtained. An observation on comparison with Figure 4.2 is that the effect of thickness on parameter distribution is not as significant as that due to sample variation.

Figure 4.4: Histogram plot of average depolarization for different sample thicknesses.

A bar plot of the average depolarization, and degree of polarization across the three samples is shown in Figure 4.5.
Figure 4.5: Bar plots of average depolarization and average degree of polarization across the samples studied.
Nonlinear imaging techniques in biomedical imaging provide many advantages in assessing and characterizing sample properties utilizing the unique peculiarities of the imaging technique. This thesis reported the methods and experimental development of second-order Mueller matrix analysis in second-harmonic generation.

Starting with a developed model for the extraction of the second-order Mueller matrix, the input Stokes vector for the identical harmonic case of the second order can be expressed as a $9 \times 1$ double Stokes vector. Hence to obtain the output representation of the Stokes vector in a conventional form, the transformation-like Mueller matrix becomes a $4 \times 9$ matrix, and this can be obtained by solving a system of equations involving known input polarization and measured output forms. Scalar matrices such as average degree of polarization and depolarization can be extracted from the output concatenated Stokes vectors.

The technique was applied to pig tendon and skin samples. Due to diversity of collagen fibers within an imaged region, the sample field of view was divided into grids and the effect of sample type, thickness and analysis grid size on scalar parameters investigated.

The two separate samples at the same thickness were studied and the variation in average depolarization parameters was obtained. Next, the same sample type at different thicknesses was investigated, and the plot of the parameters were also shown. The effect of grid size was studied by choosing grid numbers across ranges of $16 \times 16$, $32 \times 32$, $64 \times 64$ and the per pixel case of $320 \times 320$. The effect of grid size on scalar parameter distribution was shown to be insignificant above a minimum number of grids used in this thesis, and hence an optimized number can be chosen based on accuracy and computational power required.
5.1 Future Work

As a tissue assessment technique, future applications for the process involve assessing varying stages of tissue biopsy including hyperplasia, dysplasia and malignant tissue microarrays. It is expected that physical changes in the cell will yield a change in the polarization alteration properties, and by extension Mueller matrix representation, of the tissue samples. Furthermore, by taking the fiber orientation per grid into consideration, a more rigorous mathematical framework can be incorporated into the analysis. This holds promise as a multimodal approach when used in conjunction with Fourier-transform second-harmonic generation.
APPENDIX A

RELEVANT MATRIX PARAMETERS

This appendix describes some useful matrix parameters used in the Mueller matrix-based analysis.

A.1 Gell-Mann Matrices

The nine $\lambda$ matrices that form the Gell-Mann set [31] follow:

\[
\begin{align*}
\lambda_1 &= \sqrt{\frac{2}{3}} \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix}, \\
\lambda_2 &= \sqrt{\frac{1}{3}} \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & -2 \end{pmatrix}, \\
\lambda_3 &= \begin{pmatrix} 1 & 0 & 0 \\ 0 & -1 & 0 \\ 0 & 0 & 0 \end{pmatrix}, \\
\lambda_4 &= \begin{pmatrix} 0 & 1 & 0 \\ 1 & 0 & 0 \\ 0 & 0 & 0 \end{pmatrix}, \\
\lambda_5 &= \begin{pmatrix} 0 & 0 & 0 \\ 0 & 0 & 1 \\ 0 & 1 & 0 \end{pmatrix}, \\
\lambda_6 &= \begin{pmatrix} 0 & 0 & 1 \\ 0 & 0 & 0 \\ 1 & 0 & 0 \end{pmatrix}, \\
\lambda_7 &= \begin{pmatrix} 0 & 0 & 0 \\ 0 & 0 & -i \\ 0 & i & 0 \end{pmatrix}, \\
\lambda_8 &= \begin{pmatrix} 0 & 0 & -i \\ 0 & 0 & 0 \\ i & 0 & 0 \end{pmatrix}, \\
\lambda_9 &= \begin{pmatrix} 0 & -i & 0 \\ i & 0 & 0 \\ 0 & 0 & 0 \end{pmatrix}.
\end{align*}
\]

(A.1)

A.2 The Double Mueller Matrix

From Equation 3.19

\[
M_D = U^{(o)} \cdot [U^{(i)}]^{-1}
\]

(A.2)
where

$$U^{(o)} = \begin{pmatrix}
H'H' & VH + VV & PH + PV & P'H' + P'V & RH + RV & R'H' + R'V & H'H + H'V & V'H + V'V & P'H + P'V \\
HP - HP' & VP - VP' & PP - PP' & P'P - P'P' & RP - RP' & R'P - R'P' & HP - HP' & VP - VP' & P'P - P'P' \\
HR - HR' & VR - VR' & PR - PR' & P'R - P'R' & RR - RR' & R'R - R'R' & HR - HR' & VR - VR' & P'R - P'R'
\end{pmatrix}$$

(A.3)

and

$$U^{(i)} = \begin{pmatrix}
\sqrt{2}/3 & \sqrt{2}/3 & \sqrt{2}/3 & \sqrt{2}/3 & \sqrt{2}/3 & \sqrt{2}/3 & \sqrt{2}/3 & \sqrt{2}/3 & \sqrt{2}/3 \\
\sqrt{1}/3 & \sqrt{1}/3 & \sqrt{1}/3 & \sqrt{1}/3 & \sqrt{1}/3 & \sqrt{1}/3 & \sqrt{1}/3 & \sqrt{1}/3 & \sqrt{1}/3 \\
-1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 1/2 & 1/2 & -1/2 & -1/2 & 0 & 0 & 0 \\
0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
-1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
(\sqrt{2}+1)/2 & (\sqrt{2}-1)/2 & (\sqrt{2}+1)/2 & (\sqrt{2}-1)/2 & (\sqrt{2}+1)/2 & (\sqrt{2}-1)/2 & (\sqrt{2}+1)/2 & (\sqrt{2}-1)/2 & (\sqrt{2}+1)/2 \\
(\sqrt{2}+1)/2 & (\sqrt{2}-1)/2 & (\sqrt{2}+1)/2 & (\sqrt{2}-1)/2 & (\sqrt{2}+1)/2 & (\sqrt{2}-1)/2 & (\sqrt{2}+1)/2 & (\sqrt{2}-1)/2 & (\sqrt{2}+1)/2
\end{pmatrix}$$

(A.4)

The equations present the method to obtain the double Mueller matrix from measured intensities at predetermined input polarization.
APPENDIX B
POLARIZATION STATE PARAMETERS

B.1 Optical Component Angles for Experiments

Table B.1: Linear Polarizer, Quarter Wave-Plate and Half Wave-Plate Combinations for the Polarization State Generator.

<table>
<thead>
<tr>
<th>PSG State</th>
<th>LP Angle (°)</th>
<th>HWP Angle (°)</th>
<th>QWP Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0.00</td>
<td>45.00</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.00</td>
<td>22.50</td>
<td></td>
</tr>
<tr>
<td>P*</td>
<td>0.00</td>
<td>337.50</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.00</td>
<td></td>
<td>45.00</td>
</tr>
<tr>
<td>R*</td>
<td>0.00</td>
<td></td>
<td>315.00</td>
</tr>
<tr>
<td>H_P</td>
<td>0.00</td>
<td>11.25</td>
<td></td>
</tr>
<tr>
<td>V_{R*}</td>
<td>0.00</td>
<td>33.75</td>
<td>90.00</td>
</tr>
<tr>
<td>P_{R*}</td>
<td>0.00</td>
<td>326.25</td>
<td>315.00</td>
</tr>
</tbody>
</table>

Table B.2: Quarter Wave-Plate and Linear Polarizer Combinations for the Polarization State Analyzer.

<table>
<thead>
<tr>
<th>PSA State</th>
<th>QWP Angle (°)</th>
<th>LP Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td></td>
<td>90.00</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>45.00</td>
</tr>
<tr>
<td>P*</td>
<td></td>
<td>135.00</td>
</tr>
<tr>
<td>R</td>
<td>90.00</td>
<td>45.00</td>
</tr>
<tr>
<td>R*</td>
<td>90.00</td>
<td>135.00</td>
</tr>
</tbody>
</table>
B.2 PSG Stokes and Double-Stokes Representations

Table B.3: Chosen PSG States with Their Corresponding Stokes and Double-Stokes Vectors

<table>
<thead>
<tr>
<th>PSG State</th>
<th>Stokes Vector</th>
<th>Double Stokes Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H$</td>
<td>$(\frac{1}{0} \ 0)$</td>
<td>$\left( \begin{array}{c} \sqrt{2/3} \ \sqrt{1/3} \ -\frac{1}{3} \ 0 \ 0 \ 0 \ 0 \end{array} \right)$</td>
</tr>
<tr>
<td>$V$</td>
<td>$(\frac{1}{0} \ -1 \ 0)$</td>
<td>$\left( \begin{array}{c} \sqrt{2/3} \ \sqrt{1/3} \ 1 \ 0 \ 0 \ 0 \ 0 \end{array} \right)$</td>
</tr>
<tr>
<td>$P$</td>
<td>$(\frac{1}{0} \ 0)$</td>
<td>$\left( \begin{array}{c} \sqrt{3/2} \ -\sqrt{3/4} \ 0 \ \frac{1}{2} \ 0 \ 0 \end{array} \right)$</td>
</tr>
<tr>
<td>$P^*$</td>
<td>$(\frac{1}{0} \ -1 \ 0)$</td>
<td>$\left( \begin{array}{c} \sqrt{3/2} \ -\sqrt{3/4} \ 0 \ -\frac{1}{2} \ -1 \ 0 \end{array} \right)$</td>
</tr>
<tr>
<td>$R$</td>
<td>$(\frac{1}{0} \ 0)$</td>
<td>$\left( \begin{array}{c} \sqrt{3/2} \ -\sqrt{3/4} \ 0 \ 0 \ 0 \ -1 \end{array} \right)$</td>
</tr>
<tr>
<td>$R^*$</td>
<td>$(\frac{1}{0} \ -1 \ 0)$</td>
<td>$\left( \begin{array}{c} \sqrt{3/2} \ -\sqrt{3/4} \ 0 \ 0 \ 0 \ -1 \end{array} \right)$</td>
</tr>
<tr>
<td>PSG State</td>
<td>Stokes Vector</td>
<td>Double Stokes Vector</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>$H_P$</td>
<td>$\begin{pmatrix} \frac{1}{\sqrt{2}} \ \frac{1}{\sqrt{2}} \ 0 \end{pmatrix}$</td>
<td>$\begin{pmatrix} \sqrt{25/24} \ -\sqrt{1/48} \ -\sqrt{1/2} \ 1/4 \ (\sqrt{2}+1)/2 \ (\sqrt{2}-1)/2 \ 0 \ 0 \end{pmatrix}$</td>
</tr>
<tr>
<td>$V_{R^*}$</td>
<td>$\begin{pmatrix} -\frac{1}{\sqrt{2}} \ \frac{1}{\sqrt{2}} \ 0 \end{pmatrix}$</td>
<td>$\begin{pmatrix} -\sqrt{25/24} \ \sqrt{1/2} \ -\sqrt{1/4} \ 0 \ 0 \ (1-\sqrt{2})/2 \ (1+\sqrt{2})/2 \end{pmatrix}$</td>
</tr>
<tr>
<td>$P_{R^*}$</td>
<td>$\begin{pmatrix} 1/\sqrt{2} \ 0 \ -\frac{1}{\sqrt{2}} \end{pmatrix}$</td>
<td>$\begin{pmatrix} \sqrt{3/2} \ -\sqrt{3/4} \ 0 \ 0 \ -\sqrt{1/2} \ -\sqrt{1/2} \ 1/2 \ \sqrt{1/2} \ \sqrt{1/2} \end{pmatrix}$</td>
</tr>
</tbody>
</table>
REFERENCES


