Using Second Harmonic Generation Microscopy to Study the Three-Dimensional Structure of Collagen and its Degradation Mechanism

A Dissertation Presented

By

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Abstract:

Collagen is one of the most abundant proteins found in the human body. Its crystalline structure possesses no centrosymmetry, allowing it to emit second-harmonic waves. Second harmonic generation (SHG) microscopy utilizes the latter quality to produce high-resolution images of collagen rich tissues and therefore become a key research tool in the biomedical field. We developed a new model, intended to be used together with second harmonic generation (SHG) microscopy, to thoroughly investigate collagen-based tissues. We use our SHG model to reveal information in real time from enzymatic biochemical processes. We also present a novel method used to measure quantitatively the direction of the fibers within the tissue, from SHG images. Using this method, we were able to reconstruct an angular map of the orientation of collagen fibers from multiple sections across the entire area of a human cornea. The structure we obtained demonstrates the criss-crossing structure of the human cornea, previously suggested in the literature[1-3]. In addition, we also report work on a unique step-wise three-photon fluorescence excitation discovered in melanin. This unique fluorescence mechanism was exploited to discriminate melanin on a small-size, low-cost and low laser power setup which was used as a prototype for a handheld device. The latter study is a part of a larger on-going effort in our group to explore new diagnosis methods to be used for early skin cancer screening[4]. Finally, this work demonstrates a spectroscopy-based method to correct for blood vessel thickness effect. The method analyzes spectral shift from a molecular imaging agent and correlate the shifts to the length of the optical path in blood. The correction method described in this work is intended to be implemented on a guided catheter near infrared fluorescence (NIRF) intra-vascular imaging system. In
this imaging system, this study's results will be used to correct for the radial distance between the imaging tip of the catheter and fluorescing agents chemically bonded to plaques on walls of the arteries.
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Introduction

1. Introduction

1.1. Dissertation overview

This dissertation contains four major parts: (1) using second harmonic generation (SHG) microscopy to study the enzymatic degradation mechanism of collagen, (2) using SHG microscopy to map the organization of collagen fibers in human cornea, (3) enabling imaging of stepwise three photon fluorescence from melanin using a dual wedge scanning confocal microscope, and (4) molecular fluorescence imaging through blood.

The above topics are discussed in Chapters 3, 4, 5 and 6, respectively.

Chapter 2 provides an introduction to the theoretical background needed in the field of nonlinear optics and of the structure of collagen in order to assist the reader with familiarizing him/her-self with SHG microscopy for collagenous tissues.

Chapter 3 discusses a method for obtaining information regarding the progression of enzymatic degradation reaction of collagenous tissue in real-time second harmonic generation microscopy imaging. Chapter 4 describes the use of SHG microscopy to map the organization of the collagen fibers in human corneas. In this chapter we introduce a novel method of quantifying the angular orientation of fibrous features from an image. Chapter 5 presents a proof-of-concept study, showing the ability to obtain a step-wise three photon fluorescence excitation from melanin on a small scale and cheap confocal microscope setup. Finally, Chapter 6 depicts a spectral analysis based method, to correct for blood vessel thickness effects in a near-infrared fluorescence (NIRF) intravascular imaging system. This study was done in
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collaboration with Prof. Dana Brooks, Prof. Vasilis Ntziachristos and Dr. Amir Rosenthal of TUM, and Dr. Farouc Jaffer of the cardiovascular research center (CVRC) at Massachusetts General Hospital.

To date the work described in this dissertation has been published in the following:


Currently three more papers are under review, and two more papers are in the writing process. We expect to publish all of our result by fall 2013.
2. Theoretical background

2.1. Non-linear optics

Nonlinear optics (NLO) is a sub-discipline of optics that deals with the interaction between light and certain dielectric materials (known as optically nonlinear materials), in which the material’s polarization vector has a nonlinear response to the electric field of the light. The polarization vector in materials can be represented by a power series

\[ P = E^{(0)} + \chi^{(1)}E^1 + \chi^{(2)}E^2 + \chi^{(3)}E^3 + \ldots \]  \hspace{1cm} (2.1)

In this power series the first term is the DC component and is usually the result of an induced electric field. In certain cases, a rectification of the light field can leave the medium with this net DC polarization. The second term describes a normal linear response. The response of the polarization vector \( P \) will be linear to the light’s field \( E \) and will therefore have the same frequency. In optically linear materials all of the remaining terms in the equation will be non-existent. The third term is a second order process (the first nonlinear process) and its existence in a material will result in emission of second harmonic generation (SHG) waves. If the field of incident is not monochromatic, therefore contains more than one wavelength, the material could also exhibit some other second order effects such as sum-frequency mixing, difference-frequency mixing and the Pockels effect [5, 6]. Hence, second harmonic generation is sometimes considered a specific case of sum-frequency mixing, where \( \omega_1 = \omega_2 \). Similarly, the fourth term is responsible to third order
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response phenomena such as third harmonic generation, Raman effect and the optical Kerr effect.

2.2. Second Harmonic Generation

By using the polarization vector, described in Eq.(2.1), for first order nonlinear materials, it is possible to derive and expression for the expected intensity of the SHG waves.

The intensity of the second harmonic waves can be calculated by applying Eq.(2.1) into the non-linear wave equation [5, 7].

\[
\nabla^2 \vec{E} - \frac{n^2}{c^2} \cdot \frac{\partial^2 \vec{E}}{\partial t^2} = \frac{1}{\varepsilon_0 c^2} \cdot \frac{\partial \vec{P}^{NL}}{\partial t}
\]  
(2.2)

The electric fields of both the fundamental (the exciting source) and emitted SHG waves will have the form

\[
E_j(z, t) = E_j(z) e^{-i\omega_j t} + c.c. = A_j(z) e^{i\mathbf{k}_j z - \omega_j t} + c.c.
\]  
(2.3)

where \( j = 1, 2 \), with \( \omega_1 = \omega \) (the fundamental beam) and \( \omega_2 = 2\omega \) (second harmonic waves) and where c.c. stands for complex conjugate. The non-linear polarization vector will be then given by

\[
\vec{P}_2(z, t) = 2\varepsilon_0 \chi^{(2)} E_1^2 e^{i(2k_1 z - \omega_2 t)} + c. c
\]  
(2.4)

where \( k_1 = k_{\omega_1} \) is the wave vector of the fundamental.

By applying Eq.(2.4) and Eq.(2.3) into Eq.(2.2), and with some algebraic processing, the total intensity of second-harmonic waves is obtained, having the following form
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\[ I_2(L) = \frac{4\omega_2^2}{n_1^2n_2c^2\varepsilon_0} |\chi^{(2)}|^2 I_1^2 L^2 \text{sinc}^2 \left( \frac{\Delta k L}{2} \right) \]  

(2.5)

Where \( \Delta k = 2k_{\omega_1} - k_{\omega_2} \) and \( L \) is the location along the optical axis where the intensity of SHG waves is measured. It is now easy to see the existence of the phase matching condition between the fundamental wave and the emitted second harmonic wave governed by the presence of the Sinc function. The Sinc function will obtain a maximum value for \( \Delta k = 0 \), i.e. when the phases of the fundamental and emitted SHG wave are perfectly matched.

2.3. Second Harmonic Generation Microscopy

2.3.1. Overview

As suggested by the second harmonic generation equation, this non-linear optical process involves the absorption and annihilation of two photons by the medium, through which the fundamental waves are travelling, in order to generate a single photon with a doubled frequency. However SHG should not be confused with two-photon excitation fluorescence (TPEF), which is a resonant process. Fig 2-1 shows the energy diagrams for both the SHG and TPEF processes. In SHG, an electron is excited to an intermediate virtual state by absorbing a photon. While at the excited virtual state, absorption of a second photon by the electron will result in further excitation to another virtual state at a higher level. The electron will then decay back to its ground state resulting in the emission of a photon with doubled frequency.
This process can only be possible at a very high photon irradiance, since the lifetime of the virtual states is in the order of pico-seconds. In contrast, TPEF can involve an intermediate state that is either a real state, in which case the process will be referred to as step-wise TPEF, or a virtual state. The real intermediate state has a lifetime in the order of nano-seconds. In this case the emission of TPEF waves will be enabled at lower fundamental power levels. However, similar to TPEF, the power of the SHG waves will have a quadratic relationship, with respect to the power of the fundamental, as described in Eq.(2.5) for the SHG case.

As discussed earlier, the emission of SHG waves requires a non-zero second-order optical susceptibility coefficient. However, SHG waves can only be obtained from materials whose crystalline structure expresses no centrosymmetry. The


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latter is the key crystalline requirement that must be met in order to obtain second-harmonic waves from a material. Due to symmetry considerations, material that does possess centrosymmetry (meaning its crystalline structure has a center of inversion), will cause the $\chi^{(2)}$ parameter to vanish identically. Since the polarization vector has the form $\vec{P}(t) = \epsilon_0 \chi^{(2)} \vec{E}^2(t)$, as shown in Eq.(2.4), if we change the sign of the applied electric field, the term $\vec{P}(t) = \epsilon_0 \chi^{(2)} [-\vec{E}]^2(t)$ will only be true for $\chi^{(2)}=0$, forcing the second order susceptibility tensor to vanish. More detailed descriptions of the theory and models of second harmonic generation are widely available in the literature [5, 8-12].

The phenomenon of optical second harmonic generation (SHG) was first observed in the early 1960s [13] with quartz. Shortly after the discovery of the second harmonic phenomenon in quartz, SHG emission was also observed in biological tissues [14]. However, only after the development of point scanning microscope systems in the late 1990s did SHG microscopy became available as an imaging tool [15, 16]. Today, there is an increasing use of second harmonic generation microscopy as leading imaging modality for biomedical optics [17, 18]. SHG microscopy was proven to be an effective high resolution imaging technique for nonlinear biomedical tissues in general, and for collagen rich tissues in particular [19]. One of the advantages of using SHG with biomedical application is its high specificity for materials in the tissue, which satisfy the nonlinear requirement. When imaging a tissue using multiple modalities, SHG can discriminate the part of interest in the tissue with high resolution. Therefore by combining images from two or more modalities, different parts in the tissue can
be observed with high contrast. Another advantage of SHG microscopy is its ability to perform pinhole-free sectioning of the sample, giving it a three dimensional contrast mechanism similar to confocal microscopy. The confocality of SHG is made possible because of its square power dependency, previously discussed. Emission of SHG waves requires especially high irradiance levels. The high irradiance is necessary in order to assure a second photon absorption will occur during the short life time of the virtual states. For this reason, SHG waves can only be emitted from the very center of the objective's point spread function (PSF), where the irradiance of the fundamental field is at its highest level. The dependency of SHG microscopy emission in the square of the power of the fundamental, as described in Eq.(2.5), makes the PSF of SHG microscopy also be quadratically dependent on the PSF of conventional microscopy, as shown in Eq.(2.6).

$$\text{PSF}_{\text{SHG}} = (\text{PSF}_{\text{Conv}})^2$$  \hspace{1cm} (2.6)

Effectively, this reshapes the PSF of SHG microscopy, form the profile of the conventional microscopy PSF, into a spheroid, symmetric in the radial direction and elongated in the axial direction. This spherical shape is obtained by squaring the irradiance distribution in three dimensions of the PSF of conventional microscopy.
2.3.2. SHG microscopy on the Keck Fusion multimodal microscope

Fig 2-2. SHG layout on the Keck fusion multimodal microscope

The parts of this dissertation, which involved the use of SHG microscopy, were performed using the Keck 3D fusion multimodal microscope. A complete description of the microscope and its different modalities was previously reported [20, 21].

Fig 2-2 shows, the optical layout of the Keck microscope. The red line follows the optical path used for SHG microscopy. Following this beam path, the Ti:Sapphire laser, shown in the figure, uses as the exciting source. The laser light is
reflected onto a polygon mirror containing 32 sides which is used as the x-scanner. The laser then continues to the galvo scanner for a y-scan. The scanned beam then travels through a set of telescope lenses in order to adjust the field of view generated by the scanners to properly match the entrance pupil of the objective. The adjusted scanned area then enters the commercial Nikon TE2000U inverted microscope to imaging the sample. The SHG waves, emitted from the focal point of the objective from inside the volume of the sample, are travelling both in the forward and in the backward (epi) directions. The backward direction SHG signal is detected using the photomultiplier tube (PMT), which is also used by the confocal fluorescence and two photon fluorescence modalities. A second PMT (Hamamatsu H9305-01) was added to the existing setup in order to allow the detection of forward SHG signal. The forward direction detector is placed after the condenser lens. A special adapter was designed in-house to allow replacement of the condenser with a forward direction objective. The SHG signal is synchronized with the position of the scanned beam and the obtained image is displayed on a computer monitor.

2.4. The Structure of Collagen Type-I

Type I Collagen is the most abundant protein in mammals and is present virtually in every extracellular tissue with mechanical function. In tendons and ligaments, collagen transmits the force from muscles to bones and stores elastic energy. Smooth walking would not be possible without these outstanding mechanical properties. Collagen is also one of the dominant components of the organic matrix of bones and
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teeth dentin which provides them with fracture resistance. It is also a major component of skin and blood vessels and is even present in muscles. The basic building block of collagen is the protein molecule named tropocollagen. The molecule has a typical length of about 300nm and is about 1.5nm in diameter [22-24]. Collagen molecules are composed of three polypeptide alpha chains coiled around each other to form a triple helix configuration. Each individual polypeptide chain contains approximately one thousand amino acid residues. The main amino acids present in the chains are GLY (Glycine), PRO (Proline) and HYP (Hydroxyproline). The structure is stabilized by H-bonding between the different residues. This described triple helix alpha chains of collagen are shaped into a right-handed helix. Each of the Alpha chains possesses a left-handed symmetry (i.e. in the opposite direction), however the three coiled strands are twisted together to form a right-handed triple helix collagen. The molecular structure of collagen is an important factor in its ability to produce second-harmonic waves. As demonstrated before [25], the described crystalline structure of type I collagen possess no centrosymmetry, which allows it to produce SHG waves. Fig 2-3 depicts the molecular structure of tropocollagen [25].
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Fig 2-3. A chain of amino acids joined together to form the triple-helix structure (Image from the RCSB PDB (www.pdb.org) of PDB ID 1BKV [25]).

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Recent studies explored and analyzed the three dimensional crystalline structure of various types of collagen using x-ray diffraction [26] and TEM [27-29]. These studies suggest collagen fibrils are formed through an array of tropocollagen molecules staggered together in a fiber like shape and are typically about 1 to 3um in size. Similarly, a bundle of fibrils staggered together form a collagen fibers, which are typically about 5 to 20um in size. An illustration of the hierarchical structure of collagen is showed in Fig 2-4.

This unique molecular structure of collagen is responsible for its ability to produce SHG waves. The complex non-centrosymmetric crystalline structure of the tropocollagen molecule results in an efficient $\chi^{(2)}$ tensor that is very efficient in emitting second harmonic waves along the axis of the helical structure. The complete tensor as well as other symmetry considerations to the tensor is discussed later in section 3.2.1.
3. Analysis of the enzymatic degradation of collagen using SHG microscopy

3.1. Overview

Many studies in the fields of biology and biomedicine explore the details of the enzymatic degradation of collagen [30, 31]. New revelations regarding this biochemical process will contribute greatly to our understanding of the formation, growth, maintenance and diseases of load-bearing tissues [15, 27-30, 32-36]. In the past decade, the use of SHG microscopy in collagen studies has grown considerably due to the high resolution and high specificity of SHG microscopy to collagen. In addition, recent improvements in the design of environmentally-controlled bioreactors allow real-time imaging of collagen while the sample is undergoing a biochemical reaction [37].

The study reported in this chapter uses a bioreactor of this kind, together with SHG imaging, to explore the enzymatic degradation of collagen samples. In the performed experiments, a darkening of the entire image is observed as the reaction progresses. This is due to the degradation of the collagen molecules, which effectively causes the elimination of some “SHG sources”, and is pronounced by a reduction in the collected signal.

This study uses a computational model to correlate the darkening of the image seen on the microscope with the biochemical reaction taking place in the bioreactor’s chamber. The study begins with the characterization of the microscope’s point spread function (PSF) for the SHG modality. Thereafter, a three-dimensional model
of the collagen is presented which simulates the collagen sample. Next, the PSF of
the microscope is used to scan the modeled collagen structure in order to generate
synthetic SHG images. The synthetic images are then gradually degraded
computationally. Finally, the computationally degraded synthetic images are
compared with real SHG images of collagen, obtained experimentally. The study
makes use of the Michaelis-Menten biochemical model to correlate the synthetic
with the experimental results.

3.2. Characterizing the emission of SHG waves from collagen tissues

3.2.1. Deriving the point spread function (PSF) of SHG emission

In order to obtain an expression for the PSF of the SHG modality, we begin by
considering the amplitude of the source as having a Gaussian beam profile. This
consideration is justified since, in most cases, the use of an objective lens results
in a beam with a Gaussian profile at the sample.

It is necessary to begin our derivation with this assumption of the fundamental
in order to satisfy the requirement for high irradiance levels, as discussed earlier
in section 2.3.1.

Eq.(3.1) depicts the Gaussian beam profile of the fundamental. For the sake of
algebraic convenience, we use the, less-common, compact form of the Gaussian
profile.

\[ A(r, z) = \frac{A_0}{1 + i \zeta} e^{-\frac{r^2}{w_0^2(1+i\zeta)}} \]  (3.1)
Analysis of the enzymatic degradation of collagen using SHG microscopy

where $A_0$ is a magnitude parameter, $w_0$ is the beam waste and $$ \zeta = \frac{2x}{b} = \frac{2x}{kw_0^2} $$ is the unitless confocal parameter. The Rayleigh range can therefore be written as $$ b = \frac{2\pi w_0^2}{\lambda}. $$

Fig 3-1. The profile of a Gaussian beam for NA=0.45 objective using $\lambda=800\text{nm}$. (Color bar is normalized to arbitrary units between 0 and 1)

Fig 3-1 depicts the real part of beam profile intensity of a Gaussian beam for a 20x, 0.45NA objective lens using Eq. (3.1). An objective lens with parameters similar to the one characterized here was primarily used for the experimental part of SHG imaging described later in this chapter, and with the source having a wavelength of 800nm.

The SHG waves emitting from the substrate must always satisfy the nonlinear wave equation.

$$ \nabla^2 E_n - \frac{1}{c^2} \frac{\partial^2 E_n}{\partial t^2} = \frac{1}{c^2 \varepsilon_0} \frac{\partial^2 P_n}{\partial t^2} $$

(3.2)
The solution for the electric field and the polarization vector can be represented by the two phasors:

\[
\tilde{E}_n(r, t) = A_n(r)e^{i(k_n z - \omega t)} \tag{3.3.a}
\]

\[
\tilde{p}_n(r, t) = p_n(r)e^{i(k'_n z - \omega t)} \tag{3.3.b}
\]

It should be noted that Eq. (3.3.a) and Eq. (3.3.b) have a different wave vector allowing emission of different wavelengths.

It is uncommon to use SHG microscopy with high NA objective lenses. High NA objectives normally have a lower depth of focus \((DOF \propto \frac{\lambda}{NA^2})\), which requires the work with thinner samples. The loss of DOF occurs more rapidly than gain in resolution (and therefore also irradiance) \((R \propto \frac{\lambda}{NA})\), when increasing the NA, making the use of high NA objectives undesirable.

Therefore, for a low NA objective lens, we can use the slow varying amplitude approximation. The approximation is assuming the Rayleigh range is greater, by more than an order of magnitude, than the wavelength, i.e. \(Z_r \gg \lambda\). Under these conditions, the first order spatial derivative will be much greater than the second order, \(\left| \frac{\partial^2 A_n}{\partial z^2} \right| \ll k_n \left| \frac{\partial A_n}{\partial z} \right|\), which results in the “slow” change of the amplitude of the field. Since the field is propagating in the Z direction only, for convenience, we can also separate the Laplacian operator into the transverse component and the Z direction component, \(\nabla^2 = \frac{\partial^2}{\partial z^2} + \nabla_T^2\).

After applying the fields’ phasors of Eq. (3.3.a) and Eq. (3.3.b) into Eq. (3.2) and using the slow varying amplitude approximation we get
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\[ 2i k_n \frac{\partial A_n}{\partial z} + \nabla^2 A_n = -k_n^2 p_n e^{i \Delta k z} \quad (3.4) \]

where \( \Delta k = k' - k_n \) is the phase mismatch.

Substituting the \( q^{th} \) order susceptibility component into the polarization phasor we get

\[ 2i k_q \frac{\partial A_q}{\partial z} + \nabla^2 A_q = -k_q^2 \chi^{(q)} A_1^{(q)} e^{i \Delta k z} \quad (3.5) \]

In order to satisfy the diffraction theory requirements, the emitted SHG wave must also have a Gaussian profile. That is the divergence angle of any source is proportional to \( \frac{\lambda}{d} \) (where \( d \) is the size of source in the direction perpendicular to the propagation).

We can therefore write the \( q^{th} \) order Gaussian beam profile for the emitted waves, with the new addition of the \( q \) in the exponential argument.

\[ A_q(r, z) = \frac{A_q(z)}{1 + i \zeta} e^{-\frac{4 \pi r^2}{\omega^2(1 + i \zeta)}} \quad (3.6) \]

As depicted by Eq.(3.6), the newly generated photons will be emitted from a tighter beam waist, in accordance to Huygens–Fresnel principle. The expected reduction in the beam waist, dictated by the characteristics of a Gaussian beam profile, is proportional to the square root of the order of harmonic generation, which in the case of second harmonics is \( \sqrt{2} \).

We continue by applying Eq (3.6) into Eq (3.5), which results in the reduction of the order of the wave equation. We are now left with a first order differential equation that can be solved by simple integration. The field amplitude is given by
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\[ A_q(z) = \frac{iq\omega}{2nc} \chi^{(q)} A_1 J_q(\Delta k, z_0, z) \]  \hspace{1cm} (3.7)

where

\[ J_q(\Delta k, z_0, z) = \int_{z_0}^{z} \frac{e^{i\Delta k z'}}{\left(1 + \frac{2i z'}{b}\right)^{q-1}} \]  \hspace{1cm} (3.8)

is the phase-matching integral. The integral is taken over the range of the location of the nonlinear material, having non-zero second order susceptibility parameters. The phase matching term is especially important as it describes the interaction between the \( q^{th} \) order harmonic wave and the fundamental beam.

Fig 3-2 shows the normalized (arbitrary units) real part of the phase-matching integral as a function of the direction of propagation \( z \). The figure represents a slab profile which begins at \( z=0 \) and lies on the positive side of the \( z \) direction, as shown in Fig 3-3. In Fig 3-2(a) the phase matching integral is shown for a fundamental wavelength of 10\( \mu \)m and its respective second harmonic wave of 5\( \mu \)m. In Fig 3-2(b) the phase matching integral is shown for a fundamental wavelength of 800nm and its respective second harmonic wave of 400nm. The relatively long wavelength of 10\( \mu \)m is shown in Fig 3-2(a) in order to demonstrate more easily the decaying oscillation behavior of the phase matching integral.
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Fig 3-2. The phase-matching integral for a second order susceptibility material.

Fig 3-3. Profile of a material displaying a second order susceptibility along the positive part of the z direction and uniformity along the transverse r direction
For a shorter wavelength case, as shown in Fig 3-2(b) for a wavelength of 800nm, the entire positive side of the z-axis is filled with a densely oscillating curve, caused by the very short wavelength of the phase matching length, which is much smaller than the Raleigh range.

Fig 3-4(a) shows a normalized scale (arbitrary units between 0 and 1) representation of the real part of the SHG field that is emitted from the material profile described Fig 3-3 for the objective and source discussed earlier. Fig 3-4(b) shows the field intensity of the same system as in Fig 3-4(a). As described earlier, the emitted SHG pattern is emitted with a Gaussian profile. However, unlike the classic Gaussian beam case shown in Fig 3-1, the Gaussian profile of SHG carries a periodic change in the field’s intensity as a result of the phase matching condition. Some technologies take advantage of this phenomenon to constructively interfere and hence amplify the SHG signal. More detailed discussion of this technology, named quasi-phase-matched second harmonic generation, is widely available in the literature [5, 38].
Fig 3-4. A normalized (a.u.) representation of (a) the real part; and (b) the field intensity of the second harmonic waves emitted from the material profile described in Fig 3-3 with the use of a 0.45 NA objective using a source of 800nm in wavelength.

Eq.(3.7) and Eq.(3.8) are both scalar solutions to the emitted SHG fields. Therefore, in order to obtain the complete vector equation we introduce the vector field terms and by replacing the $\chi^{(2)}$ parameter with the second order
susceptibility tensor, as shown in Eq.(3.9).

\[
\begin{bmatrix}
E_x \\
E_y \\
E_z
\end{bmatrix} = \frac{i \omega f e^{\frac{-2r^2}{w_0^2}}}{n_q c (1 + i\zeta)} A_1^{(2)} J_2(\Delta k, z_0, z) \times
\begin{bmatrix}
\chi_{11}^{(2)} & \chi_{12}^{(2)} & \chi_{13}^{(2)} & \chi_{14}^{(2)} & \chi_{15}^{(2)} & \chi_{16}^{(2)} \\
\chi_{21}^{(2)} & \chi_{22}^{(2)} & \chi_{23}^{(2)} & \chi_{24}^{(2)} & \chi_{25}^{(2)} & \chi_{26}^{(2)} \\
\chi_{31}^{(2)} & \chi_{32}^{(2)} & \chi_{33}^{(2)} & \chi_{34}^{(2)} & \chi_{35}^{(2)} & \chi_{36}^{(2)}
\end{bmatrix}
\begin{bmatrix}
E_x^2 \\
E_y^2 \\
E_z^2 \\
E_x E_y \\
E_x E_z \\
E_y E_z
\end{bmatrix}
\]

For collagen imaging using SHG microscopy, it is common to reduce the tensor, described in Eq.(3.9) due to both cylindrical and Kleinman symmetry. Since the collagen molecules are staggered together to a cylindrical shape, in which the rotation of each molecule about its axis is random, the total macroscopic susceptibility result in cylindrical symmetry [39]. Kleinman symmetry refers to a condition where the frequency of the fundamental and/or emitted SHG wave is smaller than resonant electronic frequency of the material. In that condition permutation of the indices in the tensor are allowed [5].

The susceptibility tensor for type-I collagen obtained under these conditions can be written as
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\[
\chi_{\text{collagen}}^{(2)} = \begin{bmatrix}
0 & 0 & 0 & 0 & 0 & \chi_{16}^{(2)} \\
\chi_{16}^{(2)} & \chi_{22}^{(2)} & \chi_{16}^{(2)} & 0 & 0 & 0 \\
0 & 0 & 0 & \chi_{16}^{(2)} & 0 & 0
\end{bmatrix}
\]  (3.10)

The value of the tensor components where previously found [40] for type I collagen as, \( \chi_{16}^{(2)} = 0.08 \frac{pm}{\nu} \) and \( \chi_{22}^{(2)} = 0.15 \frac{pm}{\nu} \).

3.2.2. Modeling of the collagen structure

The hierarchy of the structure of collagen is discussed earlier in section (2.4). In brief, we will discuss some of the key features of the structure of collagen that have been considered for the preparation of this model.

The basic building block is the collagen molecule known as tropocollagen. The molecule has a typical length of about 300nm and is about 1.5nm in diameter. Next, the tropocollagen molecules are aggregated together in a semi-crystalline structure to form the collagen fibrils. Collagen fibrils are typically ~300nm in diameter and 1 to 20um in length. Similarly, a bundle of fibrils aggregated together in a staggered pattern to form a collagen fiber, which is typically about 1 to 5um in diameter and 10 to 200um in length.

For the computational model, we chose the size of each voxel to be 300nm x 300nm x300nm, in order to match the diameter of the collagen fibrils. This chosen voxel size is smaller, by about an order of magnitude, than the SHG microscopy resolution. Therefore, in the model, when the fibrils are staggered to form fibers, these fibers will have a size that is (a) comparable with the fibers’ size, as observed experimentally by SHG microscopy, and (b) greater than the spatial resolution of SHG microscopy. The total size of the modeled tissue in the...
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The thickness was set to be 2.1um (7 voxels) in the z direction, while the total size of the model in the x and y directions was set to be 220um. The size of the model in the z-direction is set in order to match the thickness of the sectioning resolution of SHG microscopy. Similarly, the sizes chosen for the x and y dimensions are designed to match the total field of view obtained by SHG microscopy using a 20x objective lens.

Fig 3-5 shows a cross section along the xz plane of the described model. In the figure, the black voxels represent the presence of a fiber, having the value 1, and the white voxels represent free space, having the value 0. The cross section shown in the figure is then extended along the y direction. The extension of the model along the y-direction is done using an algorithm that gives the fibers a wavy shape along the xy plane. The algorithm is designed to retain the fibers’ uniformity, i.e. a circular, 7-pixel wide, shape is kept throughout the extension of the model. The algorithm selects the amplitude, frequency, phase shift, and duty cycle of the fibers. For each of these mentioned parameters, the algorithm gets a range of values, from which the value to be used is chosen randomly.
3.2.3. Creating synthetic SHG images

The emission of SHG photons is a non-linear problem, as described by Eq. (3.9). However, once second harmonic waves are emitted, their propagation through the system’s optical components to the detector can be considered a linear problem. As shown in Eq. (3.9) and earlier in Eq. (2.5), the non-linearity describes the SHG emission in response to the fundamental field (the exciting source). In the case where the system is operating with a steady excitation source (i.e. the intensity of fundamental field remains constant over time), all the remaining terms in the above equations are constants for a given imaging system, and can be therefore combined into one constant. In addition, Eq. (3.9) and Eq. (2.5) also assume that the SHG medium is uniform.

Therefore, for constant source power conditions, the total emission of SHG is linearly proportional to the concentration of the substrate and to remaining term.
in the equation. This can be formulated into the simplified expression, as shown in Eq. (3.11).

\[ I^{SHG} = I_0^{SHG} \cdot C \cdot P_{source}^2 \]  

(3.10)

Where \( I_0^{SHG} \) is the constant per a given illumination system, \( C \) is the concentration and \( P_{source} \) is the power of the fundamental.

However, in the case where the substrate is undergoing biochemical degradation, this condition is not met. As suggested by Eq. (3.9)(3.11), changes in the concentration of the SHG-producing molecules in the substrate are expected to affect the field intensity of the emitted SHG waves linearly. This case is similar in nature to the linear process of single photon fluorescence, where the emission of fluorescing light is linearly proportional to the concentration of the fluorophore in the solution.

Since during SHG imaging the intensity of the source is fixed, the emission and propagation of SHG can be considered a linear problem. In this case, it is possible to track the progression of the emitted SHG field and to use the theory of linear systems to generate the expected SHG image synthetically.

The (PSF) was obtained using the equations derived in the previous section. Effectively, obtaining the three dimensional PSF can be achieved by rotation of the two-dimensional model, shown in Fig 3-4, about its axis. In accordance with linear systems theory, in order to obtain the emitted SHG field from the substrate, the three-dimensional PSF is convolved with the three-dimensional model, shown in Fig 3-5. Integrating the convolution result over the direction of
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propagation gives a good representation of the image obtained by SHG microscopy.

Fig 3·6. (a). SHG image of a human cornea taken using the Keck microscope; (b) A synthetic SHG image.

Fig 3·6 shows a side-by-side comparison between a synthetic and an experimentally obtained SHG image. Fig 3·6(a) shows an SHG image obtained experimentally using the Keck fusion microscope. Fig 3·6(b) shows a synthetic SHG image generated using the model and method described in this section. The most important parameter that must be compared between the two images is the size of the fibers. This is a key parameter since a mismatch could grossly invalidate the model described here. The size of the fibers was manually measured and found to match between the two images. The value found is in the order of 1 to 2um.
Fig 3-7. The distribution of fiber size in both synthetic and experimentally obtained images.

<table>
<thead>
<tr>
<th>Type</th>
<th>Number</th>
<th>Mean</th>
<th>St.dev</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp</td>
<td>50</td>
<td>2.2</td>
<td>0.5</td>
<td>2.3</td>
<td>1.4</td>
<td>3.2</td>
<td>1.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Syn</td>
<td>50</td>
<td>2.2</td>
<td>0.5</td>
<td>2.3</td>
<td>1.4</td>
<td>3.2</td>
<td>1.8</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 3-1. Quantiles of fiber size measurements. Measurement values are in μm.

The manual measurements were performed by selecting 50 random locations on both images and recording the size of the fiber at that location. The size was measured by the number of pixels a fiber had at that location. The pixel size was converted to units of μm. The numerical results as well as a graphical representation are shown in Table 3-1 and Fig 3-7 respectively. It can be seen that the sizes of the synthetically and the experimentally imaged fibers are comparable. The fibers measured at the experimentally obtained image show a slightly higher measurement values. However, as shown in Table 3-1, the quantile measurements are marginally affected by these higher values, and they can therefore be considered outliers.
Several other differences could be pointed out between the two images. However, these differences are not critical to our degradation model and analysis, since they do not challenge the validity of the proposed model. These differences in the image are a result of various artifacts that are introduced to the real image but do not necessarily originated from the sample. Some examples are the dark current of the PMT, which is temperature and gain dependent, ambient light sources and electronic noise, introduced to the I/O reading device. Excluding these artifacts from the synthetic image is preferable despite the resulting in differences it produces. The absence of these artifacts in the synthetic images does not limit our ability to draw conclusions regarding the studied degradation process. In the next section, we will use the synthetic images as a computational tool to explore and interpret structural changes of the collagen tissue.

3.3. **Interpreting of the enzymatic degradation mechanism of collagen tissues using SHG microscopy**

3.3.1. **The Michaelis-Menten model for biochemical kinetics**

The Michaelis-Menten model is one of most common models in biochemistry and it is widely used in enzymatic kinetics [41-43]. The model describes the relationship between the biochemical reaction rate and the substrate concentration as follows:
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\[ v = \frac{d[P]}{dt} = \frac{V_{\text{max}}[S]}{k_m + [S]} \]  

(3.11)

where \( v \) is the reaction rate, \([P]\) is the concentration of products, \([S]\) is the concentration of the substrate, \(V_{\text{max}}\) is the maximum rate and \(k_m\) is the Michaelis-Menten constant. \(V_{\text{max}}\) can be expressed as

\[ V_{\text{max}} = k_{\text{cat}}[E]_0 \]  

(3.12)

where \([E]_0\) is the initial concentration of the enzyme, and \(k_{\text{cat}}\) is the reaction’s catalytic constant. \(k_{\text{cat}}\), measured in units of inverse seconds, is also known as the turnover number and is defined as the maximum number of molecules of substrate that an enzyme can convert to produce per catalytic site per unit of time. \(k_m\), the Michaelis-Menten constant, is defined as the substrate concentration at which the reaction rate is at half-maximum. It also describes an inverse measure of the substrate’s affinity for the enzyme.

The Michaelis-Menten model is most accurate at equilibrium conditions, where the substrate concentration can be considered constant. In real experiment, equilibrium is not necessarily maintained throughout the duration of the enzymatic reaction. In the case of enzymatic degradation of collagen, such as the experiments reported in this chapter, equilibrium isn’t maintained at all times. The substrate concentration term in Eq.(3.12) will then be time-dependent. The complete time-dependent substrate concentration can be determined using

\[ \frac{[S]}{k_m} = W \left[ \frac{[S]_0}{k_m} e^{\left(\frac{[S]_0}{k_m} \cdot \frac{V_{\text{max}}}{k_m}\right)} \right] \]  

(3.13)
where $W(x)$ is the Lambert W function (also called the omega function), which is the solution to an implicit function of the form $z = W(z)e^{W(z)}$ and can be expressed using the series expansion $W(x) = \sum_{n=1}^{\infty} \frac{(-1)^{n-1}n^{n-2}}{(n-1)!} x^n$.

Using Eq.(3.14), it is possible to determine the substrate concentration at any point in time during the experiment. Obtaining this piece of information is of high value for our study, since it provides us with the remaining concentration of substrate at every point in time for the duration of the reaction.

### 3.3.2. Experimental results

The experimental part was done in collaboration with Prof. Ruberti’s group from the Department of Mechanical Engineering at Northeastern University. His lab uses the Nikon inverted TE2000U microscope as well. Prof. Ruberti’s group has developed a bioreactor that can be mounted on the imaging stage [37]. The bioreactor is designed to allow the biochemical reaction to take place while the sample is being imaged in real time. As discussed earlier in Section 2.3.2, the Keck microscope, used for the experiments reported here, has been upgraded with the second harmonic imaging modality, which is not available with the commercial version of the Nikon microscope.

The sample used was a human cornea obtained from Lions Eye Bank of Delaware Valley (Philadelphia, PA) that was not considered to be suitable for surgery or transplantation. The sample used was completely anonymous. The gender, age or any other detail regarding the donor was not disclosed. The cornea was kept in storage media (2.5% chondroitin sulfate dextran corneal
storage media enhanced antimicrobial system containing gentamicin sulfate 100 µg/ml and streptomycin sulfate 200 µg/ml) and was stored between 2°and 6°C from the day of the donor’s demise to the day of the experiment.

The enzyme used was bacterial collagenase. The biochemical parameters used for the reaction were: \([S]_0 = 5\mu M\), \([E]_0 = 20nM\), \(K_{cat} = 0.277 \frac{1}{sec}\) and \(K_m = 3.5\mu M\).

The total duration of the reaction time was 30 min, and the sample was imaged throughout the reaction. Each image was taken using an average of 64 frames.

The total time interval between each image was approximately 5 seconds. At the end of the experiment, a total of 390 frames were taken.

![Graph](image)

Fig 3·8. Theoretical curve of the remaining substrate concentration as a function of time, for the biochemical parameters used for the experiment described in this section. As shown by the intersecting dashed lines, at the completion of the experiment time \(\cdot\) 30 min \(\cdot\) the remaining fiber concentration was determined to be 39 percent of its initial value.

The curve shown in Fig 3·8 depicts the expected concentration of the remaining substrate as a function of time. The curve was obtained using the theoretical model for the biochemical kinetics described in Eq.(3.14) with the parameters
values listed above, which were used in this experiment. Upon completion of the experiment, at a time of 30 minutes, the expected concentration of the remaining fiber was 39% of the initial concentration.

Fig 3-9. SHG images, taken in real-time, from the sample while the enzymatic degradation was taking place. The images were taken at times: a) 0 min; b) 6 min; c) 12 min; d) 18 min; e) 24 min; f) 30 min.

Fig 3-9 shows a series of images taken in real-time during the progression of the biochemical reaction. The images shown here were taken with 6 minutes intervals from the same location in the sample. As can be seen, a darkening of the images is observed as the reaction progresses. This darkening effect is expected, since the enzyme effectively disintegrates the collagen molecules. This effectively eliminates some of the sources emitting the SHG waves, resulting in a weaker signal to be collected by the detector.

In order to mimic the experimental degradation process with the synthetic images, the 1 value that is set to the three-dimensional model in Fig 3-6 is replaced by a fractional value, which represents the concentration of the
remaining tissue. The justification for doing so lies in the fact that the biochemical degradation occurs at the molecular level, which is far smaller than the optical resolution. In other words, the chemical “etching” of a single (or even several) molecule cannot be directly resolved. However, the total signal strength that is read from the location where the reaction took place will weaken. This weakening will be pronounced by the darkening of the pixel, which is related to that location. And since energy is a scalar, the total energy of the SHG waves, which is detected at each pixel, will be proportional to the substrate’s concentration. Therefore, the brightness of the image is also proportional to the concentration of the substrate.

Fig 3-10. Synthetic SHG images simulating the conditions of enzymatic degradation at substrate concentration a) 100%; b) 86%; c) 72%; d) 58%; e) 44%; f) 30%.

Fig 3-10 shows a series of the synthetic images that are computationally degraded. Similar to the real SHG images, a darkening effect can be observed as the substrate concentration is reduced.
3.3.3. Comparison between model and experimental results

In this section we will compare the degradation observed during the real enzymatic process, with the computationally generated process. Since for both sequences the degradation is observed by a darkening of the image, we chose the brightness of the image as a parameter for comparison between the computational and the experimental results. As discussed at the end of section 3.3.2, the relationship between the concentration and the brightness of a pixel is assumed to be linear.

![Graph showing comparison between simulated and experimental collagen degradation](image)

Fig 3-11. A comparison between simulated and experimental collagen degradation

For each of the degradation processes, the computational and the experimental, the darkening effect at each time step was measured by taking the average of the brightness level of the pixels in the image divided by the average of the image brightness of the first image, when the degradation process started.
Analysis of the enzymatic degradation of collagen using SHG microscopy

\[ C = \frac{\sum_i^n \sum_j^m P_{i,j}}{\sum_i^n \sum_j^m P^0_{i,j}} \]  

Eq. (3.13) shows the concentration calculation used in this part, where \( C \) is the concentration, \( P_{i,j} \) is a pixel value of the time step image and \( P^0_{i,j} \) is the pixel value of the first image in the sequence.

In order to ensure that the image intensity isn’t affected by outlier pixel values, the mean value was compared with the median. For both the experiment and simulation a good match is found between the mean and median, indicating no significant outlier pixels were present.

Fig 3.11 shows a comparison between the simulated image sequence and the experimental sequence. As can be seen, the experimental degradation curves show good match to the computationally degraded curves suggesting the model can provide a good prediction of the degradation stage. The curves produced by the real measurements have two points where over-time there is an increase in the brightness of the image. This is believed due to relaxation and shrinkage of the collagen structure by the reaction and dehydration. The mechanical movement of collagen molecules during the shrinkage can shift molecule into the imaged field of view, therefore resulting in a slight increase in the local concentration and brightness.

3.4. Conclusions

The computational model described in this chapter can provide the user with information regarding the concentration of the substrate as well as the reaction rate, by examining images taken during the occurring reaction. The analysis can
either be performed after the reaction is completed, or can be integrated into the imaging system for real time measurement, which can help to monitor the reaction. The described method can be used to study structural relationship in collagenous tissues, since the core of model lies on the structural hierarchy of collagen. However, with minor alteration, it is also possible to utilize and extend this model to study other materials and tissues.
4. Mapping the orientation of collagen fibers in the human cornea

4.1. Overview

The cornea is a vital component in the eye’s mechanical structure and has a great effect on its optical functionality. The mechanical roles of the cornea include providing a frontline layer of protection from injuries, maintaining the ocular pressure and withstanding the forces of the extra-ocular muscles during eye movement. Its optical role requires the cornea to be transparent to visible light and to have a precise curvature in order to support its functionality as the preliminary eye lens. The cornea’s shape, being spherical near the visual axis and flattened at the periphery, is specifically designed to address the latter requirement. The shape of the cornea as well as its mechanical and optical properties, are derived from the specific arrangement of its collagen lamellae. The cornea is composed of mainly water and collagen types I, III and V [44, 45], with type-I collagen being predominant. In addition to the mentioned fibril-forming collagen types, there are some non-fibril forming components, including collagen type VI and XII [46]. The cornea is composed of several sections along the optical axis, yet the layer that is of most interest is the stroma. The stroma makes up ~90 percent of the entire corneal thickness, and most of the fibrous collagen is found in this layer. The stroma has a layered structure where sheets of collagen fibers, or lamellae, cross parallel to the surface of the cornea rather than through its thickness [2, 3, 47]. Several studies have shown that the organization of the collagen lamellae in the center of the cornea corresponds to the orientation of the superior-inferior and nasal-temporal directions.
Mapping the orientation of collagen fibers in the human cornea

[1, 48, 49]. Other studies showing correlation between the tensile strength of the collagen fibrils and their alignment with the direction of stress [50-54] have led to the hypothesis that fibrils in the center of the human cornea adopt a preferred orientation necessary to resist the mechanical forces of the four rectus muscles [55, 56]. The limbal annulus region is located at the interface between the cornea and sclera, providing additional reinforcement [57, 58]. In the past decades, studies using the X-ray diffraction method have made a great contribution to our understanding of the structure and organization of the cornea [1, 3, 48, 50, 51]. However, with new advances in the field of second harmonic (SHG) microscopy, the orientation of collagen lamellae can be quantified more accurately, since the high-resolution images produced by SHG can provide a visual and detailed validation to the study. We will use this approach in this chapter to explore the organization of collagen in the human cornea. The results of this study provide further insight into understanding the structure of collagen and into the relationship between structure and functionality.

Several recent studies reported the development of a robust technique to quantify the orientation of the fibers from SHG microscopy images [59-61]. One of these methods, developed in our group, is a key tool for this study and will be discussed in detail in the next section.

4.2. An algorithm for finding the orientation of fiber from SHG images

Several techniques have been investigated for SHG images in an attempt to quantify the orientation of the fibers in a lamella of a cornea. Normally, traditional
image processing tools, such as the Radon or the Hough Transforms would be a suitable solution for the problem [62, 63]. However, SHG images of collagenous tissue often comprise many lamellae crisscrossing with crimps of different shapes and amplitudes. With the use of traditional techniques, this crimped structure makes finding the orientation a challenging task. This is because both Radon and Hough transforms (commonly available in image processing toolboxes) are designed for finding straight lines in an image. One of the ways previously used addressed the problem by taking advantage of the properties of forward-emitting SHG waves. The forward direction SHG photons are emitted when the orientation of the fibers in the lamellae are aligned with the direction of the polarization vector of the fundamental [39, 64]. The use of this method is rather qualitative, since the angular resolution is fairly limited. Other techniques employ manual methods combined with a supporting analytical method, such as the Fourier Transform, to validate the result obtained during the process [65-67]. However the manual methods lack consistency, since these often provide different results when performed by different users. In addition, as we will discuss later, manual analysis of SHG images can often bias the results because it allows the user’s personal interpretation. Furthermore, when dealing with a large data set, such as is often collected from a Z-stack or an orientation mapping across a large area, manual selection of the orientation can be both tedious and very time consuming. Other commonly used techniques employ x-ray diffraction [3, 68, 69]. However the results obtained by x-ray lack the imaging aspect, making it harder to validate the results. Additionally, when using x-ray diffraction, the information obtained represents an integration of the diffraction
Mapping the orientation of collagen fibers in the human cornea

pattern over the entire thickness of the sample, therefore lacking the axial depth resolution. Finally, x-ray based techniques are also not immune from fibrous crimps, and the results are likely to be affected by it.

We present here a novel technique to quantify the different orientations of the collagenous lamellae in the cornea consistently and robustly. Although this method can be used to quantify the orientation of other fibrous substrates, we focused our effort on SHG images from corneal stroma. This method is found to be particularly useful for SHG images where the fibers have a highly crimped architecture. The method was first tested on synthetically generated images, where the angle, amplitude, thickness, frequency and the duty cycle of the crimpy fibers in the image are provided. The results obtained by this method were then compared to the input parameters. The results showed an excellent match between the orientation found by the method’s algorithm and the input values. Determining the direction of the fibers in a crimpy lamella, especially in the case where the crimps are of a high amplitude, can be quite challenging, since the wavy slope of the crimps can be mistakenly considered a distinguished direction. The direction of the crimps, however, is of no real value and we are interested, rather, in the overall orientation of the fibers.

The processing steps discussed in this chapter were performed using MATLAB® 2011(a), however they can be implemented using any other image processing software.
As discussed earlier, in order for the method to be effective, it is necessary to address the challenge of reducing the sensitivity to the crimps in the fibers. Fig 4·1 shows an example of a typical SHG image from a human cornea. The image shows obvious wavy fibers that are all oriented at an angle close to, yet smaller than, 90 degrees (vertical lines in the image). The crimps are small, changing their angle by only a few degrees. Fig 4·2 shows the steps of the method used by our algorithm in order to obtain the angular information.

![SHG image from a human cornea](image_url)

**Fig 4-1.** An example of a typical SHG image from a human cornea.

We begin the process by performing a fast Fourier transform (FFT) to the image from which we are trying to obtain the angular information. Fig 4·2(a) shows the FFT image of Fig 4·1, where the brightness of the FFT image is displayed in logarithmic scale. Displaying the FFT image in logarithmic scale is necessary, since the 0th order is ~5 orders of magnitude greater than the next highest value. Displaying the same image in linear scale will appear as a single white dot at the center of the image. As can be readily seen in the figure, the original image contains information in wide distribution of spatial frequencies along the direction
orthogonal to the lamellae seen in Fig 4-1. The FFT image has a property that we are looking to take advantage of, which is that all recurring features in the image (including the fibers), regardless for their value or position in the original image, are now pronounced by lines running through the center of the FFT image. However, the crimps seen in the original image will also appear on the FFT image at the location of spatial frequency of the fibers. To avoid accounting for the crims’ frequencies, we apply a Band Pass Filter (BPF), 0.035-0.573 [um⁻¹] to the FFT image, as shown in Fig 4-2(b). The two dashed yellow circles represent the locations of the cutoff frequencies of the BPF. The use of the BPF serves a dual purpose, since it removes both the DC background noise as well as the high-frequency fibrous information.

In the next step, we filter the remaining FFT image by adjusting the contrast, as shown in Fig 4-2(c). This operation is done by adjusting the histogram such that the lower level is set to twice the minimum value of the original histogram, while the original maximum remains unchanged. This latter step might change from one optical system to another, depending on the background level or other artifacts in the image. The adjustment of the histogram helps to eliminate the low value integrals in the Fourier Transform, which do not contain spatial information of real value about the sample. These eliminated frequencies are a result of noise in the real image due to artifacts such as the ones discussed earlier in section 3.2.3. We proceed by applying a median filter with a size of 4 x 4 pixels. The median filter is used to remove “salt-and-pepper” noise from the remaining FFT image, which is scaled due to the histogram adjustment, as shown in Fig 4-2(d). The image is then
rotated by 90 degrees to correct for the $\pi/2$ angular shift between information in the real and the Fourier domain (this step is not shown). In the next step we perform a Radon Transform (RT) to the FFT image displayed in Fig 4-2(d). The result is shown in Fig 4-2(e). The dominant direction in the FFT shows a clear peak along the x' = 0 in the RT image. In the RT image, a peak suggests the presence of a straight-line feature in the image that underwent the transform. Therefore, a high value on RT line x' = 0 indicates the high presence of fibers at that angle. The results are shown in Fig 4-2(f). As shown in the figure, the method indicates that the fibers in the examined lamella are oriented at an angle just shy of 90 degrees. This result shows agreement with a manual measurement preformed on the image in Fig 4-1. An example of a typical SHG image from a human cornea Fig 4-1.

The width of the peak shown in Fig 4-2(f) represents the angular variation of the fibers within a lamella at a single orientation. This variation is a result of the actual angular variation of the lamella and is not affected by the direction of the crimps in the fibers. The shape of the peak in Fig 4-2(f), its width and the total angular distribution of Fig 4-2(f) are comparable to some previously reported studies that investigated the angular information of the cornea’s structure using the X-ray diffraction method.
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Fig 4-2. The steps in the algorithm used to quantify the orientation of the fibers. (a) Log scale FFT image; (b) Applying a Band Pass Filter to the FFT image; (c) Contrast adjustment; (d) Applying a median filter; (e) Radon Transform; (f) taking the x’=0 the Radon transform.

However, the real strength of this method is its ability to analyze images containing several strands of collagen oriented at different directions.

Fig 4-3(a) shows an example of such case where there are several orientations present in the image. The image was processed using the method described and the results are shown in Fig 4-3(b).

The different directions found in Fig 4-3(b) are marked using arrows with different colors. The arrows are also showed on the original image in Fig 4-3(a), to indicate
the location in the image where the fibers are oriented in that found direction. As shown, for each of the peaks obtained Fig 4-3(b) a corresponding location was readily found in the original image with strong fibrous presence.

It should be noted that in cases where the lamellae in the image contain multiple directions, similar to Fig 4-3(a), it will be more difficult for an observer to perform a manual independent measurement. In such cases, since an observer is less sensitive to variations in the brightness of the features, the result will be greatly influenced by structural information. However, a measurement of that kind isn't necessarily accurate, especially in the case of SHG imaging, where a brighter SHG signal suggests a higher concentration of collagen fibers in the imaged location.

Furthermore, with every measurement, an observer will perform a certain level of image processing. By trying to measure the direction of the fibers, the observer will have to first determine the path of the fibers. This result might change from one observer to another, especially for low-contrast images. Therefore a method that is sensitive to structural changes can provide biased results and will be more sensitive to contrast changes.
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Fig 4.3. A lamella containing several different orientations of fibers (a), and the corresponding angular result obtained using our technique (b).
4.3. Mapping the orientation of fibers in human cornea

We will now use the algorithm described in section 4.2 to map the orientation of the collagen fibers across the entire human cornea.

4.3.1. Experimental

Our experiments were performed using the Keck fusion multimodal microscope, as described earlier in section 2.3.2. The samples were excited using 800nm wavelength and a narrow band pass filter—Thorlabs FB400-10 400±2nm—was placed in front of each of the backward and forward PMT modules. The imaging process was done with a Nikon Plan Apo microscope objective: 20x • 0.75 NA. Two human cornea samples, approximately 11mm in diameter, were used. The human corneas were obtained from Lions Eye Bank of Delaware Valley (Philadelphia, PA) and were not considered to be suitable for surgery or transplantation. The corneas were kept in cornea storage media (2.5 percent chondroitin sulfate dextran corneal storage media enhanced antimicrobial system containing gentamicin sulfate 100 μg/ml and streptomycin sulfate 200 μg/ml) and were stored between 2 and 6 degrees Celsius from the day of the donor’s demise to the day of the experiment. The samples used were completely anonymous. The gender, age or any other detail regarding the donor was not disclosed. All cornea samples were prepared for the imaging using the same following method. On the day of the experiment, the corneal tissue was removed from storage media and placed onto a clean coverslip. The scleral tissues were cut from cornea using clean razor blades and then washed with DI water to remove all debris. After washing with DI water, the cornea samples were placed
on a new, clean coverslip (No. 1.5) and a few drops of DI water were added to delay dehydration. A second coverslip was placed over the sample, in order to hold the sample in the center and to keep the sample as flat as possible, to assure it is perpendicular to the imaging plane across the whole surface of the cornea. The prepared sample with the coverslips was then mounted to the invented microscope’s stage to be imaged.

Four z-sections were taken from both samples. For the first sample, 22 by 22 images were taken at each z-section with steps of 5um to create a mosaic representation. For the second sample, 21 by 30 images were taken for each z-section with steps of 2um along the z direction. The 22 x 22 sample was designed to have an equal sampling rate in x and y, while the 21 x 30 sample was designed to have an equal sampling area (since each image has a field of view of 280 x 220um with 640 x 480 pixels). Fig 4-4 shows the mosaic made of the 21 x 30 images of the second sample stitched together to form a complete representation of the cornea. A total of four z-sections were chosen as the maximum number of slices to be imaged, since the imaging time for each slice is approximately 6 hours, after which the samples begins to dehydrate and shrink.
4.3.2. Data Processing

Each of the images from the mosaic in Fig 4-4 was processed using a novel algorithm described in detail in section 4.2. As described, the algorithm provides a final result in the form of a plot, where for each discrete angle value between 0 and 180, a value in arbitrary units that represents the presence of fibers in that direction is obtained.

The data from the quantification algorithm is then re-represented using a polar plot. The polar plot representation is obtained by duplicating the data points of 0 to 180 degrees to the data points of 181 to 360 degree. The latter is justified since a result of either 45 degrees or 135 degrees represents a fiber pointing in the same direction. The polar plots are used to replace the individual SHG images of Fig 4-4. We will refer to the processed mosaic, composed of polar plots, as
“direction mosaic.”

The representation of the direction mosaics was processed further in order to allow easier observation of the crossing lamellae. Improvement of the direction mosaics was done by adjusting the size and/or color of each polar plot. The adjustment of the size and color is done by one of two approaches: max value normalization or max area/integral normalization. In the max value normalization for each z-section slice, all the data values from the quantification algorithm are aggregated from all the images across that same slice, and are then normalized between 0 and 1. Using this normalization approach, higher values from our algorithm are considered to have a stronger and more pronounced presence of fibers in that direction and are, therefore, weighted accordingly. In addition, in order to avoid scaling while accounting for outliers, the distribution of the direction values was taken for each z-section and the 65th percentile value was used to replace the max value for the scaling, after it was found to be the most adequate, empirically. Fig 4·5(a) shows the distribution of the direction values of the z-section mosaic shown in Fig 4·4. The distribution shows a bi-modal behavior with a long tail toward the higher values. This behavior was typical to all z-sections from both samples, as well as other samples (not included in this work). In the max integral/area normalization, the area of the plot obtained from the quantification algorithm is calculated for each SHG image. Then, the maximum area value across the z-section is assigned the value “1,” while the lowest area is assigned ‘0.’ The distribution of the integrals/areas values of Fig 4·4 is shown in Fig 4·5(b). The distribution shows a bi-modal
behavior with a wide separation between the two modes’ values. In the case of the max integral normalization approach, the distribution presented in Fig 4-5(b) was also typical of all samples and z-sections. A possible explanation for the similarity of the distributions across samples and z-sections is discussed later.

Fig 4-5. The distribution of direction values (a) and algorithm’s plot areas taken per SHG image (b) of the z-section shown in Fig 1.

4.3.3. Results

Fig 4-6 shows four direction mosaics taken from the first human cornea sample. The direction mosaics were taken with 5um steps along the z-direction. The color of each polar plot in the mosaics was normalized using max value normalization.
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Fig 4-6. Four z-sections of the first human cornea sample taken at z location s (a) 0um; (b) 5um; (c) 10um; (d) 15um. The color of the polar plots was scaled using maximum value normalization.

The direction mosaics in Fig 4-6 show support for the hypothesis of the woven-like lamellae structure. Starting from Fig 4-6 (a), we can see that the lamella at the top-right of the figure is growing in area as we proceed deeper into the sample. It is shown by the growth of the yellow region on the top-right part of the mosaic. In addition, an alternating yellow and red region is observed at the left side of Fig 4-6(a). The dominant direction of the fibers is alternating in correspondence to the alteration in color.

Fig 4-7 shows four direction mosaics taken from the second human cornea
sample. The direction mosaics were taken with 2μm steps along the z-direction. Two dominant colors are apparent in the mosaics: red and blue. The presence of two dominant colors is in compliance with the distribution of the max area/integral normalization, previously discussed in Fig 4·5(b).

In order to examine if there is a relationship between the red and blue regions of Fig 4·7 and the direction of the lamellae present in that location, a direction filter was applied. A direction filter switches off any desired range of angles, by assigning a zero value to these angles in the data obtained from the quantification algorithm. An examination of the direction distribution of the second sample was performed in order to find the dominant directions numerically. The distribution showed two dominant directions of the lamellae with 40 degrees and 150 degrees (not shown). The filter was then applied correspondingly with a tolerance angle of ±5 degrees.
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Fig 4-7. Four z-sections of the second human cornea sample taken at z locations (a) 0um; (b) 2um; (c) 4um; (d) 6um. The color of the polar plots was scaled using max area/integral normalization.

Fig 4-8 shows in four steps the process that was used to examine the relationship between max-integral normalization value and direction. Fig 4-8(a) shows the unfiltered image of Fig 4-7(c). The yellow color in Fig 4-8(a) has no significance and was simply chosen for the convenience of good image contrast. Fig 4-8(b) and Fig 4-8(c) show the filtered 150±5 degree and 40±5 degree image of Fig 4-8(a), correspondingly. A yellow color was again chosen to represent the 150-degree filter and light blue was chosen to represent the 40-degree filter. The colors in
these two figures were chosen for contrast purposes. Fig 4-8(d) shows an overlapping image of the two figures Fig 4-8(b) and Fig 4-8(c).

Fig 4-8. The four steps of the process examining a correlation between the direction and spatial location. (a), an unfiltered image of Fig. 4(c). (yellow was chosen for good contrast). (b) 150±5 degree and (c) 40±5 degree direction filter applied to (a) (yellow and a light blue were chosen for contrast purposes only). (d) Overlapping image of (b) and (c).

It can be seen qualitatively from the overlapping figure that there is a correlation between the fibers’ direction and their spatial location. Regions containing very little to no presence of the 150-degree filter showed significant presence of the 40-degree filter, and vice versa. This behavior was typical to all the mosaics of Fig 4-7, which were processed using the direction filter approach.
Fig 4-9 shows the four z-sections of Fig 4-7 after they were processed using the 150±5 degree and 40±5 degree direction filters, as described by Fig 4-8. Similar behavior in Fig 4-8(d) can be observed in all four filtered directions mosaics.

![Fig 4-9. Four z-sections of Fig 4-7 processed using the 150±5 degree and 40±5 degree direction filters, as described by Fig 4-8.]

**4.4. Conclusions**

The presence of two main colors, yellow and red, in Fig 4-7 is in agreement with the discussed distribution of direction values from Fig 4-5(a). It is also apparent, qualitatively, that the alternating locations of Fig 4-6(a) have alternating dominant direction. Given the sectioning resolution of SHG microscopy of ~5um, this suggests
a woven organization of the lamellae, where the lamella from below the focal plane is brought into and out of the plane of focus. The growing area at the top-right side of Fig 4-6(b), as we continue to move deeper into the sample in the z-direction (as shown in Fig 4-6(c) and Fig 4-6(d)), suggests we are observing a different lamella that is brought to the focal plane. The lamella’s uniform direction and color provide supporting evidence to the latter interpretation. The uniformity in color indicates similar gray brightness of the original SHG image. Since the image brightness is related to the amount of collagen present in the focal plane, uniformity in the color can indicate a relationship to a certain lamella, since a lamella tends to keep its consistency along the cornea [1, 48, 49]. In Fig 4-7, similar to Fig 4-6, there is a good agreement to the distribution of the max area/integral normalization as depicted in Fig 4-5(b). It is clear that the blue and red regions are matching the high and low area distributions in Fig 4-5(b). The analysis presented in Fig 4-8 and Fig 4-9 shows correlation between the spatial location and the direction of the fibers. In addition, by comparing Fig 4-7 and Fig 4-9, it can be seen that the red regions in Fig 4-7 match the locations in Fig 4-9 with dominant presence of fibers directed at 40 degrees. Similarly, the blue regions in Fig 4-7 match the locations in Fig 4-9 with dominant presence of fibers with 150 degrees. This supports our previously mentioned discussion which suggests that different lamellae have different consistency and orientation with a woven organization going in and out of the plane of focus. The latter results and interpretation are in agreement with the work previously reported in the field using the x-ray diffraction method. We are currently exploring new ways to prevent the dehydration of the sample in order to achieve
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sampling throughout the entire stack of the corneal stroma and perform a full comparison to the x-ray diffraction method.
5. Three-photon fluorescence of melanin with a dual wedge confocal scanning system

Melanoma is one of the most commonly occurring cancers in the United States and in the western world. From 1975 to 2009, although the overall cancer mortality rates have decreased by nearly 40 percent in men and by 75 percent in women (between 1992 and 2009, compared to between 1975 and 1992), the mortality rates of melanoma showed a much slower decline [70, 71]. One of the major challenges with melanoma is early clinical diagnosis [72]. Presently, the most widely used method by dermatologists for clinical evaluation of melanoma is visual inspection using epiluminescence (dermoscopy). However, this technique isn’t robustly reliable and some studies have rated its sensitivity at under 85 percent [73-77]. The current leading and most reliable method for melanoma diagnosis remains biopsy and histopathology. However, due to lack of standard practice between different hospitals and due to the subjective diagnosis of different pathologists, a large rate of discord was found among pathologists for melanoma diagnosis [78]. The low reliability rate of current methods leads doctors to err on the side of caution: leading to an increase in the number of false-positive diagnoses and to increased medical costs, emotional trauma, and insurance difficulties resulting from unnecessary surgeries, biopsies, and other treatments.

Despite the difficulty in diagnosis, melanoma is still believed to be a promising target for optical diagnosis. First, lesions suspected to be cancerous are accessible optically, making the imaging problem relatively simpler. Second, the two dominant types of melanin, eumelanin and pheomelanin, whose characteristics in the lesions play an
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important role in the diagnosis of the lesion, have the potential of producing an intrinsic molecular contrast when imaged.

Eumelanin is a dark brown/black pigment and has photoprotective and antioxidant properties. It is the pigment responsible for dark hair color, and in the skin it acts as a protective component against UV radiation. Pheomelanin is reddish yellow and exhibits phototoxic and pro-oxidant behavior [79]. It is more common in people with red hair and is particularly concentrated in the lips, nipples, glands of the penis, and the reproductive organs. Since melanin’s presence can indicate the metabolism and location of melanocytes and melanogenesis in tissue, the spatial distribution of both eumelanin and pheomelanin could assist in identifying high-risk lesions. Previous studies found that in malignant melanoma lesions, the amounts of eumelanin are considerably higher than pheomelanin [80, 81]. The latter findings inspired other researchers to explore a method for specific detection of melanin. In a recently published study, researchers tried to characterize the spatial locations and distribution of eumelanin and pheomelanin in an attempt to develop a new method for early diagnosis of lesions [82].

Previous work in our lab has shown that the visible emission from melanin is strong and can be observed easily with a near-infrared continuous-wave (CW) laser using low power [83]. This is due to a unique step-wise (SW, discussed in Section 2.3.1) three-photon excitation of melanin. The work discussed in this chapter shows that the previously reported SW, three-photon fluorescence can also be observed with the use an inexpensive CW laser using a dual-prism scanning apparatus. This demonstrates the potential of the technology to be integrated into a portable confocal microscope for
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clinical diagnosis applications. The results presented here are in agreement with those previously obtained using a larger and more expensive femtosecond laser system.

5.1. Confocal microscopy

Confocal microscopy was first introduced in the late 1950s by Minsky [84] as a subsurface imaging modality. At the heart of the method is the use of a pinhole in front of the detector to eliminate out-of-focus information. Fig 5·1 shows the optical layout for a confocal microscope. The use of a pinhole in front of the detector requires collection of the image information one focal spot at the time. In this mode of operation, namely point scanning systems, it is common to use either a set of two synchronized Nipkow discs with a wide field illumination [85], or to use a laser source with a scanning apparatus [20]. However, the requirement to scan the sample point by point inherently slows down the image acquisition process, in comparison with full-field, where the entire image is acquired in one time step. The improvement in image quality resulting from the use of the technology can be assessed in terms of the imaging system’s PSF.

\[
h(u, v) = \int_0^1 J_0(v \cdot \rho) e^{-\frac{i u \rho^2}{2 \rho}} d\rho
\]

Eq.(5.1) shows the expression for the PSF of an imaging system in optical coordinates [86]. In Eq.(5.1) \(J_0\) is the 0th-order Bessel function, \(A\) is the amplitude of
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the field and $\rho$ is the radial coordinate. The optical coordinates $u$ and $v$ represent
the axial and the lateral directions, respectively, and are given by

$$u = \frac{2\pi NA^2}{\lambda n} \cdot z$$

(5.2.a)

$$v = \frac{2\pi}{\lambda} NA \cdot r$$

(5.2.b)

Since the amplitude given by Eq.(5.1) cannot be measured directly, it is common to
describe the PSF by the its intensity distribution:

$$I(u, v) = \left( \frac{2\pi}{\lambda} \frac{NA^2}{n} A \right)^2 \left| \int_0^1 j_0(v \cdot \rho)e^{-\frac{i\rho^2}{2}} \rho d\rho \right|^2$$

(5.3)

By looking at one of the variables at the center of the PSF, we can obtain the
familiar expression often found in the literature:

$$I(v) = \left[ \frac{2J_1(v)}{v} \right]^2$$

(5.4.a)

$$I(u) = \left[ \sin \left( \frac{u}{4} \right) \frac{u}{4} \right]^2$$

(5.4.b)

According to the Rayleigh criterion, the lateral resolution of an optical system is
defined as the width between the origin $v$ and such that $J_1(v) = 0$, or $v = 3.83$ which
reduces Eq.(5.4.a) and (5.4.b) to their well-known form:

\[ \Delta x = 0.61 \frac{\lambda}{NA} \]  
\[ \Delta z = \frac{2n\lambda}{NA^2} \]

However, in the case of confocal microscopy, since both the detector and sample are in an image plane, the total point spread function of the illumination system, as shown in Eq.(5.1), must be multiplied by one another as shown in Eq.(5.6).

\[ \text{PSF}_{\text{confocal}} = \text{PSF}_{\text{detector}} \times \text{PSF}_{\text{illumination}} \]

Since the pinhole is effectively an aperture, in the Fourier domain it will have an airy disk shape similar to that of the illumination system’s PSF. Therefore, it is acceptable to approximate the PSF of the pinhole as equal to that of the illumination system. The resolution terms obtained for confocal microscopy will therefore be

\[ I(v) = \left[ \frac{2J_1(v)}{v} \right]^4 \]
\[ I(u) = \left[ \sin \left( \frac{u}{4} \right) \right]^4 \]

or the real coordinates systems from
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\[ \Delta x = 0.44 \frac{\lambda}{NA} \]  

\[ \Delta z = 1.52 \frac{n\lambda}{NA^2} \]  

(5.8.a)  

(5.8.b)

Using the expressions given in Eq. (5.5.a)-(5.5.b) and (5.8.a)-(5.8.b), it is possible to evaluate both the axial and lateral resolution. Therefore, the improvement in the axial resolution is estimated as \( 1 - \frac{\Delta z_{\text{confocal}}}{\Delta z_{\text{conventional}}} = 26\% \); similarly, the improvement for the lateral resolution can be estimated as \( 1 - \frac{\Delta x_{\text{confocal}}}{\Delta x_{\text{conventional}}} = 32\% \).
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Fig 5·1. Optical layout of a confocal microscope. The objective lens focuses the light from a point at the sample plane to a spot at the detector plane. Out-of-focus points are focused to planes before and after the image plane. The light from the out-of-focus planes is blocked by the pinhole and only in-focus information is therefore collected.

Confocal microscopy has two main sub-modalities: confocal reflectance and confocal fluorescence. In confocal reflectance, as suggested by its name, the contrast mechanism is governed by reflectance. Reflectance will occur every time the light
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traveling through the material encounters a change in the index of refraction. This type of reflection, also known as Fresnel reflection, is the main contributor to the reflected light collected by the detector in confocal microscopy.

Confocal reflectance grew as one of the promising modalities for melanoma diagnosis. The great potential for working with confocal microscopy in skin is, in part, due to the highly reflective components in skin tissue. In human skin, some of the highly reflective skin components include melanin (n = 1.72), hydrated collagen (n = 1.43), and keratin (n=1.51). These components will appear bright with good contrast when surrounded by the epidermis (n=1.34) and dermis (n =1.41), which make up the bulk of skin tissue.

The other sub-modality in confocal microscopy is confocal fluorescence. In confocal fluorescence, the contrast mechanism is governed by the fluorescing components. These components fluoresce due to an intrinsic interaction with the exciting source, i.e. auto-fluorescence, or as a result of a bonding to a fluorescing agent. Since single-photon fluorescence, which is the type of fluorescence usually referred to in the context of confocal fluorescence, is a linear process, the emission of fluorescing light will occur from the entire volume of the PSF of the system. Therefore, the pinhole is necessary in blocking the out-of-focus light in order to allow sectioning.

5.2. Dual-wedge scanning system

Most of the point-scanning confocal microscopes generally use a raster scanning system, which is easy to control and provides and sequential scan of field-of-view. However, these scanning systems often use mirrors to deflect the beam, which means a deflection of the beam off its optical path. This approach, often referred to
as “off-axis,” naturally result in an increase of the total size of the system. The dual-wedge [87, 88] scanner discussed in this chapter is an on-axis method that is based on the Risley prism approach. Being an “on-axis” approach, the dual-wedge scanner has the potential of reducing the total size of the optical system, therefore allowing it to fit into a handheld device.

![Diagram](image)

Fig 5.2. Demonstration of the dual-wedge scanning apparatus. (a) A single prism deviates the beam by the vector \( \mathbf{v}_1 \), and (b) two prisms deviate the beam by the vector sum \( \mathbf{v}_1 + \mathbf{v}_2 \). (c) A circle is scanned about the deviation from the first prism when the first prism is stationary and the second prism is rotated about the optical axis.

Fig 5.2 demonstrates a walk-through of the operation of the dual-wedge’s scanning apparatus. Fig 5.2(a) shows the operation of a single prism with a vertex angle \( \alpha \). The total deflection depends on the index of refracion of the prism and the vertex angle \( \alpha \). The beam will be deflected by a vector \( \mathbf{v}_1 \), which can be calculated easily using
Snell’s law. Fig 5-2(b) shows the total deflection caused by placing two prisms. The total deflection is going to be the sum of the deflections caused by each of the prisms. This case can be considered equal to having one prism with a vertex angle that is the sum of the vertex angles of both prisms. Fig 5-2(c) shows in four steps the scan operation caused by the rotation of one prism. In this case, the first prism remains stationary, while the second rotates about its axis. This results in a circle with a radius $v_2$ around the deflection position caused by the first prism. The total deflection of the system can be found by tracing the rays using

$$\vec{V}_{j+1} = \frac{n_j}{n_{j+1}} \vec{V}_j + \sqrt{1 - \left(\frac{n_j}{n_{j+1}}\right)^2 \left(1 - (\vec{V}_j \cdot \vec{N}_j)^2\right) - \frac{n_j}{n_{j+1}} \vec{V}_j \cdot \vec{N}_j} \vec{N}_j$$

(5.9)

where $j$ is the surface sequence, $\vec{V}_j$ is a unit vector parallel to the incident beam, $\vec{V}_{j+1}$ is parallel to the refracted beam, $\vec{N}_j$ is normal to the surface and $n_j$ is the index of refraction of medium $j$.

The scanning pattern obtained by the scanner is dependent on the ratio of the rotational speed of the two wedges. If the prisms are rotating in the same direction but at different rotational speeds, a spiral scan pattern will be produced, as shown in Fig 5-3(a). In this, the rotational speed difference causes the effective vertex angle to change over time, resulting with a convergence and divergence of the pattern toward the center and circumference, respectively.
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If the prisms rotate in opposite directions, a rosette scan will be produced, similar to the pattern shown in Fig 5·3(b).

![Fig 5·3. Scan patterns produced for (a) co-rotation and (b) counter-rotation of the prisms at different speeds](image)

Fig 5·4 show the layout of the dual-wedge confocal microscope. The laser field travels along the optical axis and is scanned by the dual-wedge apparatus. The set of telescope lenses adjust the field of view of the scanned pattern to match the entrance pupil of the objective. The reflected light is un-scanned and transferred to the detector. The quarter-wave plate assists the polarizing beam splitter in discriminating between light travelling to the sample and reflected light traveling back from the sample. A pinhole is placed in front of the avalanche photo diode (APD) to block out-of-focus light, as described earlier in Section 5.1. The dichroic mirror in the image plane between the two telescope lenses is used to discriminate fluorescing light and reflected infrared light. The specific parameters and models of the components are described in detail in the experimental part in Section 5.4.
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5.3. The stepwise three photon fluorescence process

The stepwise three-photon fluorescence process discovered earlier in our lab [83] is slightly different from other multi-photon excitation processes often used in biomedical imaging. When looking at the absorption spectrum of materials, the dark lines, as demonstrated in Fig 5-5, represent real states. The energy of the absorbed photon, inversely proportional to wavelength of the dark line, satisfies the electron's eigenfunction. On the other hand, the energy level associated with a virtual state, would not satisfy the electron's eigenfunction. The effective meaning derived from this is that no measurement of any system would show that this state is occupied.
Fig 5-5. An illustrated absorption spectrum demonstrating the presence of several real states in the visible spectrum.

This discovery of the virtual state was discovered in 1931 by Maria Göppert-Mayer [89]. In her paper she describes the virtual state as a superposition of states, however it is not a true eigenstate of the atom or molecule [7, 90]. The individual energy transitions from the ground state to the intermediate state, and also the transition from the intermediate state to the final state, are considered non-energy conserving transitions. That is, a photon's energy, absorbed by an electron, isn’t expressed in any physical way by the absorbing material. However, once the process is completed, the energy is conserved between the initial state and the final state. The lifetime of virtual states is very short in comparison with real states. The virtual states’ lifetime is in the order of picoseconds, while for real states the lifetime is often in the order of or longer than nanoseconds.

Fig 5-6 shows the energy diagrams of both the stepwise three-photon fluorescence and for the three-photon excitation fluorescence (TPEF) process. As shown in Fig 5-6(a), in order to obtain three-photon fluorescence emission, the two photons must be observed while the electrons are in the virtual state. The use of the term fluorescence also suggests the emitted light has a red shift with respect to the total energy absorbed. This is shown both in Fig 5-6(a) and (b) as the small reduction in the energy level. The lost energy is converted into thermal energy, which is expressed as an increase in the energy of the vibrational or rotational mode of the
absorbing molecules. The remaining energy is then released as a red-shifted photon. Since the lifetime of the virtual state in TPEF is very short, for such absorption to occur, very high levels of irradiance are required. Due to the proximity between the lifetime of TPEF and the laser’s pulse time, the power of the emitted fluorescence light will be proportional to the average of the cubic of the pulse laser power, \( F \propto \langle I^3 \rangle \).

In the case of stepwise absorption, the lifetime of the two real states allow work with much lower irradiance levels. Therefore, in this case of the stepwise process, the power of the emitted fluorescence light will be proportional to the cubic of the average laser power, \( F \propto (\langle I \rangle)^3 \). The two power dependencies may seem mathematically similar, however the power levels required to produce TPEF are greater by approximately 10 orders of magnitude than the stepwise process:

\[
\frac{\langle I^3 \rangle}{(\langle I \rangle)^3} \approx 10^{10}.
\]
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![Energy diagram](image)

Fig 5.6. Energy diagram demonstrating (a) three-photon excitation fluorescence; (b) Stepwise three-photon fluorescence,

In practical multiphoton microscopy systems, due to the short lifetime of the virtual states, only work with a femtosecond pulse laser can actually produce three-photon excitation fluorescence. As demonstrated later in the chapter, the stepwise fluorescence process can be observed with low power CW lasers.

5.4. Experimental

To test for the stepwise three-photon fluorescence on the dual-wedge setup, we used pure samples of melanin from Sepia (M2649), purchased from the Sigma Chemical Company. The melanin sample is in the form of dry black powder, which was spread on the surface of a R3L3S1N Negative 1951 USAF Test Target (Thorlabs, Newton, NJ). The powder was held in place by placing a number-one microscope cover slip on
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top of it. The use of the USAF target as a background substrate served the purpose of assuring good registration of the location of the melanin particles while switching modalities. However, a unique pattern substrate is not necessary for the experiment. Individual granules of melanin could be observed. They ranged in size from less than a micrometer to tens of micrometers and were mostly spherical or ellipsoidal in shape. The second sample that was tested consisted of strands of black human hair, about 50 micrometers in diameter, mounted directly on an EIA Gray-Scale Pattern Slide (Edmund Optics, Barrington, NJ). The purpose for using the gray-scale pattern as a substrate was to provide a background pattern that will assist in finding the focal location while imaging the hair sample, whose wavy shape was moving in and out of focus.

The dual wedge scanning confocal microscope, described in section 5.2, was used to image the samples. The optical layout of the microscope is described in Fig 5-4. The system used an M-x20 0.4 NA Newport objective, The dichroic mirror transmits wavelengths longer than ~750 nm and reflects shorter ones. The source laser uses a continuous wave (CW) laser diode with 24mW of power operating at wavelength of 839nm. The use of the dichroic mirror allows the system to operate while using both of the modalities, in which both confocal reflectance and stepwise three-photon fluorescence images can be obtained simultaneously. The PMT used was a Hamamatsu 3907-03, (Hamamatsu, Bridgewater, NJ) which displays a fairly high sensitivity into the near infrared (NIR) region. The high sensitivity of the PMT in the NIR region can pose a problem for imaging using fluorescing light. Since the laser power is several orders of magnitude greater than the fluorescence power,
even a small fraction of laser light, reflected from either the sample or the surfaces of the optical components could produce a higher signal than the three-photon fluorescence light. In this case, the specificity of the system to the three-photon fluorescence process would be severely impacted, which would invalidate the results. To avoid this problem, an additional 800nm short-pass filter was added in front of the PMT. The filter had an optical density of 5.32 at 839nm and was verified adequate for the task. The filter’s operation was verified sufficient, since the PMT did not show a response to direct exposure of the laser. The latter test confirmed any signal displayed by the PMT will be the result of fluorescent light. In addition, a pinhole 1mm in diameter was added in front of the PMT. The purpose of the relatively wide pinhole is to limit the amount of ambient light entering the PMT side, rather than to provide confocality. Although the experiments were performed in a dark room, it is believed that light from the LEDs of the supporting electronic equipment is sufficient to cause a false read. As described earlier, the PMT was stationed in a pupil plane. This location was optimal for collecting the fluorescent signal since, in a pupil plane, the beam is stationary in the focal point, which minimizes the loss of the fluorescent light.

5.5. **Results**

The laser power at the sample was measured to be ~0.5mW. This power level is sufficient for activating the melanin particles in order to obtain the enhanced melanin fluorescence emission [83]. Fig 5-7 shows a series of images obtained from the confocal reflectance mode, the three-photon fluorescence modalities and the merged image of the two modalities (Fig 5-7(a), 5-3(b), and 5-3(c), respectively). In
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Fig 5·7(a), the melanin particles appear, which suggests that strong reflection of the NIR light can be observed. This strong reflection of melanin was previously reported [91] and is a result the high difference in the index of refraction compared to the surrounding medium. In Fig 5·7(b), the background information is completely suppressed and only fluorescence from certain areas containing melanin is observed. From Fig 5·7(c), it can be seen that the regions in the image that exhibit high three-photon emissions are perfectly registered with the position of the melanin particles in the confocal reflectance image.

Fig 5·7. Images of enhanced emission of Sepia melanin in the atmosphere. (a) confocal reflectance image. (b) three-photon image. (c) merged image. Scale bar is 10µm.

Similarly, Fig 5·8 shows a series of images obtained from a dark human hair. The images are ordered similarly to Fig 5·7. As can be seen from the images, in the case of the human hair as well, the regions exhibiting fluorescence are perfectly registered with the position of the hair sample. No fluorescence is apparent in regions where the hair is not present. The confocal image, Fig 5·8(a) and the
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stepwise three-photon emission in Fig 5-8(b) are slightly misplaced along the optical (or focal) axis. The confocal reflectance image is focused on the substrate, while the fluorescence image is focused on the center of the hair. This intentional offset of the focal plane of the reflectance image to the substrate causes the hair to appear dark, giving high contrast to its position.

Fig 5-8. Images of a dark human hair in the atmosphere. (a) confocal reflectance image. (b) three-photon image. (c) merged image. Scale bar is 10um.

5.6. Conclusions

The phenomenon of stepwise three-photon fluorescence obtained from melanin, observed earlier in our lab using a femtosecond pulsed laser, was also observed using a CW laser and a dual-wedge confocal system. The results show excellent agreement with those obtained earlier using the pulsed laser. The results suggest that using this dual modality imaging method (i.e. confocal reflectance and stepwise fluorescence) makes it possible to identify melanin and its spatial distribution within a tissue. It is the intention of future work to obtain similar images from skin tissue, ex-vivo. Observing the stepwise fluorescence using the dual-wedge
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microscope also demonstrates that it is feasible to obtain similar results with the use of a low-cost CW laser and a simple scanning apparatus. This combination of a melanin-specific imaging technique assembled into a low-cost device has great potential to become an early skin cancer diagnosis technique.
6. Using spectral measurements to correct for blood vessel thickness effects

Atherosclerosis is one of the leading causes of morbidity and mortality globally, particularly in developed societies [92]. Recent studies have shown that inflammation is an inherent contributor to atherosclerosis, from its inception and through its progression, which ultimately leads to thrombotic complications [93, 94]. Therefore, early detection of atheroma inflammation plays an important role in preventing such life-threatening events [95-97]. Depending on the progression stage of the plaque buildup, the cardiologist can decide on the appropriate treatment. This includes lifestyle modification, pharmaceutical inhibitors, or a surgical solution such as stenting and coronary bypass [98]. Currently, many of the imaging approaches for atherosclerosis focus on the anatomic and physical characteristics of vessel narrowing [99-102]. However, these approaches lack the information necessary to gain insights regarding the plaque progression. In-vivo molecular imaging is a promising modality that can provide cardiologists with reliable information regarding the progression stage of the inflammation. By using the appropriate imaging agent [103, 104], it is possible to determine the stage of the inflammation and recommend the appropriate treatment accordingly. One specific method that was found especially efficient for this purpose is near-infrared fluorescence (NIRF). In the recent years, NIRF has emerged as a powerful technique for intravascular imaging due to some advantages that are unique to it. NIRF provides a high intrinsic sensitivity (picomolar range) and the possibility to work with a broad array
of attachment chemistries for targeted and activable agents. In addition, NIR light
offers efficient work with the transmissible optical window. Finally, the work with
NIRF makes the translation to a clinical setup easier with NIRF catheters already
available to OCT as well as FDA-approved fluorophores [105-108].
However, the information obtained by NIRF-based catheters does not include a way
to determine the distance from the tip of the catheter to the walls of the arteries,
making it difficult to distinguish between a high fluorescence signal, which is a
result of a higher inflammation level, and a high signal due to a closer proximity of
the catheter to the fluorescing plaque. We present here a method that offers a way
to resolve axial distance between the tip of the catheter and the wall of the artery
using spectral methods.
The main idea behind obtaining depth resolution from spectral data is that the
spectrum of the fluorescing light can vary, upon its arrival to the detector,
depending on the optical properties of the intermediate medium. This concept has
been adopted recently by bioluminescent tomography systems to obtain depth
resolution of targeted cells [109-113]. Since the optical absorption and scattering
coefficients of blood are wavelength dependent [114, 115], the different wavelengths
of the fluorescing light’s spectrum will be absorbed and scattered accordingly,
resulting with an altered spectrum to be collected by the detector.

6.1. Model
The ability to resolve the distance between the fluorescing source and the detector
through a blood medium was first modeled and simulated. The spectrum of the
AF750 fluorophore was obtained from the vendor. The wavelength dependency of
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the optical properties of 100 percent oxygenated blood was extracted from previously reported work [114, 115] and is shown in Fig 6·1.

![Graph showing wavelength dependency of optical properties](image)

Fig 6·1. The wavelength dependency of the optical properties of 100 percent oxygenated blood

The original data obtained from the literature included the absorption, scattering and anisotropy coefficients. The reduced scattering coefficient was calculated using Eq.(6.1.a), where $\mu_s$ is the scattering coefficient and $g$ is the anisotropy coefficient, representing the average cosine of the scattering angle $g = \langle \cos \theta_s \rangle$, and is therefore a measure of the asymmetry of the scattering function.

Next, the reduced transport coefficient was calculated using Eq.(6.1.b). The reduced transport coefficient represents the average number of scattering and absorption events that a photon undergoes per unit of length. Since both the absorption and scattering events reduce the photon’s probability to escape the medium and arrive
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at the detector, $\mu'_t$ is also often referred to as the reduced attenuation coefficient.

This is especially true if the medium’s scattering components have preferred backward-scattering small angles, where $g$ has a value close to -1.

Finally, the total reduced attenuation coefficient was used to calculate the transport scattering mean free path as demonstrated in Eq. (6.1.c).

$$
\mu'_s = \mu_s(1 - g) \quad (6.1.a)
$$

$$
\mu'_t = \mu_a + \mu'_s \quad (6.1.b)
$$

$$
l'_t = \frac{1}{\mu'_t} \quad (6.1.c)
$$

The model then predicts the attenuation for every wavelength by simply using Beer’s law as shown in Eq. (6.2).

$$
I(\lambda) = I_0(\lambda) \cdot e^{-\frac{d}{l'_t(\lambda)}} \quad (6.2)
$$

Using this approach, we obtained the predicted spectrum, for any depth of blood, through which the original spectrum travels.
6.2. Experimental

The experimental portion of this work included two main parts. In the first part, a feasibility check was performed by measuring the shifts of LED with a known spectrum. The LED that was used—LED780E (Thorlabs, Newton, NJ)—has an emission spectrum similar to that of the clinically approved NIRF imaging agent, Prosense VM110 (excitation/emission, 750 nm/780 nm, VisEn Medical, Woburn, MA). The spectra from the LED were recorded through various path lengths of blood. The blood sample used was freshly drawn, 5mL, 100 percent oxygenated blood taken from a rabbit. An anticoagulant agent was added to the sample and the experiment time was kept under two hours at room temperature in order to minimize possible changes to the optical properties of the blood. The LED experimental setup is shown in Fig 6-2(a). The blood was held in a small, clear plastic bag and was fixed against a flat, clear piece of glass. The LED light travelled through the blood and exited at the other side of the plastic bag. An optical fiber was mounted on a micropositioner and was moved to the desired optical path length by poking into the blood bag. The spectrum was recorded using USB4000-VIS-NIR (Ocean-Optics, Dunedin, FL). The second part of the experiment was performed using fluorophore instead of the LED. An AF750 fluorophore (Alexa-Fluor 750 excitation/emission, 750 nm/780 nm Invitrogen/Life technologies, Grand Island, NY) was used with a concentration of 3μM-producing optical density of 0.4 for a wavelength of 750nm. A 10mW 750nm CW laser was used to excite the fluorophore. Fig 6-2(b) shows the experimental setup using the fluorophore. The fluorophore was held in a cuvette, in place of the flat glass. In order to block the laser’s signature, a
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760 long pass filter was added before the spectrometer. The use of fluorophore as a source required careful handling. Even minor changes to the fluorophore led to photobleaching and affected the emitted spectrum. The exposure times of the fluorophore to ambient light and to the exciting laser were, therefore, carefully monitored, confirmed identical for the different runs. In addition, a fresh fluorophore from the same batch was used for each of the experiment runs in order to minimize the undesired effect on the results.

Fig 6-2. The experiment setup (a) LED experiment; (b) Fluorophore experiment.
6.3. Results

6.3.1. Model simulation

Fig 6·3(a) shows the expected spectra derived from the original spectrum. Each of the colored curves represents a different depth step between 0 and 3mm, where the original spectrum is represented by the 0mm depth. All curves showed in the figure were normalized to have values between 0 and 1. As can be seen, the original spectrum undergoes a blue shift with the increase of blood depth. The full width at half maximum (FWHM) was measured for each of the spectra in Fig 6·3(a). The locations of the two wavelengths having the FWHM value were recorded. The average of these two wavelengths, representing the center point of the FWHM on the wavelength axis, is calculated and used as the metric for the spectral shifts. This location will be referred to as the FWHM middle-point in the remainder of this chapter. Fig 6·3(b) shows the expected position of the FWHM middle point as a function of the optical path length (or depth) in blood. The spectra show a fairly linear blue shift of approximately 4nm for 3mm of optical path length in blood.
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Fig 6-3. The model used to resolve depth in blood. (a) Location of the middle point of FWHM as a function of the depth of blood; (b) The normalized spectra of AF750 for several depths of blood.
6.3.2. LED results

Fig 6.4 shows the results obtained from the LED experiment. Fig 6.4(a) shows the spectra of the LED through various depths of blood similar to the curves shown in Fig 6.3(a). The LED spectra exhibits similar behavior of a blue shift as predicted by our model.

Fig 6.4. LED results. (a) Location of the middle point of FWHM as a function of the depth of blood; (b) The normalized spectra of LED780E for several depths of blood.
Fig 6·4(b) shows the total blue shift of the FWHM middle point as a function of the optical path length through depth of blood, similar to the curve shown for the model in Fig 6·3(a). Each of the the 10 curves in Fig 6·4(b) was obtained from a separate experiment run, where each run produced its own series of spectra curves, such as the ones shown in Fig 6·4(a). As in the model, the curve shows a fairly linear blue shift with ~4nm shift for 3mm of optical path length in blood.

6.3.3. Fluorophore results

Fig 6·5(a) shows the spectra obtained from the excited AF750 fluorophore through various depths of blood. Similar to the model results in Fig 6·3 and the LED results in Fig 6·4, the spectra exhibit a blue shift with an increase of optical path length in the blood. During the experiment, the spectra obtained by the fluorophore AF750 experiments had a significantly lower intensity, due to a much lower emission (in comparison to the LED). To make up for the lower intensity, the averaging time of the spectrometer was increased from 2 sec to 35 sec. In addition, due to scaling of the background noise by the normalization, a 15 data-point median filter was applied to the final spectra. Fig 6·5(b) shows the post processing curves.

Similar to the data presented in Fig 6·3(b) and Fig 6·4(b), Fig 6·5(b) shows the FWHM middle point as a function of the optical path length in blood. Each of the the 4 curves in Fig 6·5(b) was obtained from a separate experiment run, where each run produced its own series of spectra curves, such as the ones shown in
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Fig 6.5(a). The curve shows a fairly linear blue shift with ~ 4nm shift for 1mm of optical path length in blood.

Fig 6.5. Fluorophore results. (a) Location of the middle point of FWHM as a function of the depth of blood; (b) The normalized spectra of AF750 for several depths of blood.
6.4. Conclusion

The results obtained by our experiments suggest that it is possible to retrieve the depth of blood, through which the light was travelling, by measuring its spectrum. The trends shown in Fig 6-5(b) indicate a slope of \(~4\text{nm/mm}\), i.e. 4nm of blue shift to the middle point of the spectrum’s FWHM, per mm of distance in oxygenated blood. The results from the fluorophore AF-750 experiment showed a higher level of noise due to an insufficient sensitivity of the detector used. A comparison with the results obtained by the LED experiment suggests that with the use of a more sensitive spectrometer the signal-to-noise ratio can be dramatically improved and a higher resolution of the distance through blood can be achieved. The data presented here shows great agreement with our model, as described earlier. Together with work done previously in the field \([109\text{-}113]\), our experiments provide supporting evidence for the possibility of using the described approach with other applications having a diffusive medium. However, the great the potential, it should be pointed out that the spectra of fluorescing agents can be affected by changes in the fluorophore’s local environment \([116\text{-}119]\). In these cases, the changes to the spectra as a result of the local environment can increase the uncertainty of the measurement or even invalidate the result completely. It is our intention to explore the possibility of integrating the findings of this study into a correction method on our NIRF system.
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Bibliography


