Using SHG Directionality to Characterize Collagen Alteration in Breast Cancer Tumor Microenvironment and Its Prognostic Applications

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USING SHG DIRECTIONALITY TO CHARACTERIZE COLLAGEN ALTERATION IN BREAST CANCER TUMOR MICROENVIRONMENT AND ITS PROGNOSTIC APPLICATIONS

By
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A THESIS

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Although breast cancer is a growing health concern worldwide, the challenge to minimize mortality rate partly comes from the heterogeneity in its pathological characteristics. The tumor microenvironment is a complex hub of signaling cascades that plays a key role for the progression of cancer to develop to metastatic stage. The extracellular matrix (ECM), as a major component of the tumor microenvironment, contains signatures that have cues to understand tumor progression. Here, the unique microstructural collagen alterations specific to reactive stromal/tumor cell interactions and interactions of reactive stromal fibroblasts with different tumor cell types MCF7A and MDA-MB-231 were investigated. Early alterations of collagen were characterized using the label free, collagen specific, multiphoton laser scanning imaging modality known as Second Harmonic Generation microscopy (SHG). The directionality effect of SHG, calculated as the ratio between the forward and backward SHG ($F_{SHG}/B_{SHG}$), is used to characterize the different collagen signatures locally at 10-pixel (3.52μm) dilation from the cell boundaries. Activated fibroblasts remodel collagen differently resulting significantly higher $F_{SHG}/B_{SHG}$ than deactivated fibroblasts and Wilcoxon rank-sum test gives $p<0.05$ showing the
statistically significant difference. The gels where stromal activated fibroblast and MCF7A cancer cells were interacting resulted in a significantly lower $F_{SHG}/B_{SHG}$ ratio than collagen gels where stromal deactivated fibroblast were co-cultured with MCF7A cancer cells. The Wilcoxon rank-sum test shows the statistically significant difference ($p<0.05$) in their $F_{SHG}/B_{SHG}$ ratio. The remodeling effect of MDA-MB-231 cancer cells when they interact with stromal activated fibroblast had skewed higher $F_{SHG}/B_{SHG}$ ratio than when interacting with deactivated fibroblasts. The MDA-MB-231 samples do not have a statistically significant difference ($p>0.05$) in their $F_{SHG}/B_{SHG}$ ratio.
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INTRODUCTION

The tumor microenvironment is a complex hub of signaling cascades. Stromal cells are 80% of the tumor microenvironment and tumor microenvironment is 90% of the solid tumor mass. The organization of collagen fibers in the tumor microenvironment plays a significant role in tumor progression. Cancer Associated Fibroblasts (CAFs) in the tumor microenvironment are involved in modifying ECM protein and providing cancer cells with structural signaling. CAFs are also involved in inducing organizational changes in collagen fiber microstructure. Understanding the underlying mechanism of fiber organization that contributes to metastasis is crucial. The interaction between stromal and tumor cells in reactive and normal conditions give an in depth understanding of ECM remodeling that aids tumor metastasis. In this thesis the questions asked include are their unique microstructural collagen alterations specific to reactive stromal/tumor cell interactions? Digging deeper, are the interactions of reactive stromal fibroblasts dependent on the tumor cell type? To answer these questions the stromal/tumor cell interactions were modeled by the in vitro collagen gels prepared as described in the following section.

Advancements made in modern microscopy especially nonlinear optics made it possible to carry out different biological research. The nonlinear optical process that is used to characterize the different collagen microstructural alterations quantitatively is second harmonic generation microscopy (SHG). SHG is a collagen specific multiphoton imaging technique in which signals are generated from the intrinsic properties of collagen molecules. The directionality effect calculated as the forward/backward (F_{SHG}/B_{SHG}) ratio is used to analyze remodeled collagen quantitatively.
Biological image analysis in recent years has been advancing to allow the extraction of the maximum information from a biological sample image. Image segmentation as part of the image analysis, is used to separate features in the image of the biological sample for region of interest or bulk analysis. In this thesis work an image analysis pipeline is developed to segment cell fluorescence images and to calculate the $F_{SHG}/B_{SHG}$ ratio at regions of interest. This thesis provides a simple image analysis technique that couples pixel-by-pixel cell segmentation with object segmentation that can be adopted easily. Additionally, the FIJI macro that was made in this thesis presents a simple way to quantitatively assess specific regions of interest without needing to worry about background pixel values. Ultimately, this work provides the outline of challenges in breast cancer, interactions of different stroma/tumor cell, image analysis developed to quantify the biological change, and review for the possibility in SHG commercialization for clinical use.
CHAPTER 1
BACKGROUND

1.1 Breast cancer worldwide

Breast cancer is the second leading cause in cancer related death and the most dominant type of cancer in female. According to a 2020 report by GLOBOCAN and the World Health Organization (WHO), breast cancer has the highest incidence rates of all cancers in 159 of the 185 countries observed. Moreover, WHO ranked all kinds of cancers to be the current leading cause for a minimal life expectancy in many countries worldwide. The worldwide 2020 cancer record shows that out of the estimated 19.3 million new cancer cases, 24.5% of it is comprised by breast cancer and from almost 10 million cancer death female breast cancer contributes 15.5% of the incidence (see Figure 1). In the United States, in particular, the American Cancer Society has promoted early cancer detection campaigning using simple self-examination and clinical breast exam since 1930s. Following the recognition of mammography as a breast screening technique in the 1950s, the American Joint Committee in Cancer determined staging classifications. The increased early cancer detection campaign improved the access women had for advanced clinical breast examination. The escalation of breast cancer diagnosis since the 1980s, increased the number of diagnosed women and showed a very small change in decreasing the mortality rate. Interestingly, the highly promoted early detection techniques did not significantly reduce the mortality rate in the US as the graph from the analysis on the incidence and mortality rate between black and white female shows (see Figure 2). This difference in survival between the two race comes from access for early detection, follow up rates, and pathological differences in the breast cancer subtype. The probability of black female being diagnosed with triple negative breast cancer subtype and being diagnosed with beyond stage I breast cancer resulted in a higher mortality rate than white female.
Figure 1: The 2020 GLOBOCAN Pie Chart on the most common cancer types in female represents the total number of new cases (A) and deaths (B) recorded worldwide. Again in 2020, breast cancer accounts for majority of both the new case (2.3 million) and death (0.68 million) records out of all the different cancer types. Breast cancer has shown persistency in being leading cancer incident around the world. \(^2\)
Figure 2: Female breast cancer incidence and mortality rate studies for White (including Hispanic) females vs black (including Hispanic) females between 1975-2018 shows that incidence rates for White female are higher than Black female however, the mortality rate for Black female is higher by 39% than White female. In general, mortality rate has not shown change in the rate since throughout the years of diagnosis.¹⁰

Globally WHO organized several early detection campaigns to minimize the total number of young and adult women that are dying from breast cancer. The report released by GLOBOCAN in 2018 shows the number of incidence and mortality rate for women age groups of 45+ and 45-in high, high middle, low middle, and low income countries.¹² As shown in Figure 3, higher income countries have increased number of women that get diagnosed in both age groups compared to low income countries. However, the death record for both age groups is consistent in all income countries. Furthermore, in that same year, WHO started promoting early diagnosis and clinical breast examination through screening for all women worldwide, especially in
developing countries for better patient prognosis.$^{13}$ Along with this early detection campaign, WHO also implemented step by step plan for clinical based examination and treatment. Nonetheless, in low middle- and low-income countries where it is difficult to perform these strategies routinely, it is hard to make any population-based claim on the efficiency of the early cancer detection techniques. Moreover, in these developing countries the coupling effect of early detection and treatment has on breast cancer incidence is limited by several factors. Whereas in higher income countries where women have access to routine screening and other clinical breast examination, the efficiency of early breast cancer detection campaign is visible through the increased incidence rate (Figure 3).

Figure 3: The 2018 GLOBOCAN age adjusted rates of breast cancer analysis worldwide comparing incidence and mortality rates among older age (45+) (A) and younger age (<45) (B) female shows consistency in the mortality rate in all the different countries of varied income. However, incident rates are higher for both age groups in high income countries than low-income countries.$^{12}$
Although breast cancer is a growing health concern worldwide and several early detection methods are being advertised, it is very heterogeneous in its pathological characteristics.\textsuperscript{14} Breast cancer, in general, develops in several stages as shown in Figure 4A. The stages are classified clinically based on tumor size (T), nodal status (N), and metastasis (M). TNM classification is used to determine the degree of the cancer spread from local to distant using either pathological stages (performed by examining a tissue removed during surgery) or clinical staging (based on physical exam, biopsy, or screening).\textsuperscript{15,16} The survival rate for women with breast cancer varies with the different stages. Studies showed that out of all breast cancer cases 20\%-30\% are most likely to grow into a metastatic stage and $>90\%$ of breast cancer related death is caused by secondary metastasis.\textsuperscript{17,18} The American Cancer Society, in its 2021 report presented the 5-year relative survival rate for the different stages as shown in the table in Figure 4B. In some breast cancer cases if the women are able to get the needed diagnosis and treatment, they have a better prognosis unlike some breast cancer cases that do not have a promising prognosis despite the treatment. Prognosis rate for women depends on the breast cancer subtype in which the poorest survival rate is seen among women with triple negative subtype.\textsuperscript{19}
Figure 4: Stages of breast cancer (A) and the survival rate at the different stages (B)\textsuperscript{20}. Survival rate decreases as the breast cancer develops into the metastatic stage. (C) breast cancer survival percentage for a period of months depending on the metastatic site. \textsuperscript{18}
The Breast Global Health Initiative developed a framework for middle- and low-income countries to have access to better treatments following clinical diagnosis to minimize mortality from late-stage breast cancer.\textsuperscript{21–24} Despite the work to improve breast healthcare in these developing countries factors ranging from economy to cultural beliefs still this day have implications on the survival rate in many areas. The promotion to educating women on developing the habit for early age breast examination and better life style in developing countries has been recognized as a crucial tool to reducing the number of women diagnosed for late stage breast cancer.\textsuperscript{25} On the other hand, in developed countries there is an increased rate in availability of breast cancer treatments for each stage and every breast cancer subtype. The adequate detection and treatment resources that women in developed countries have access to, significantly improved the 5 year survival rate.\textsuperscript{26} Contrarily, in developed nations overdiagnosis and overtreatment has been a growing concern for the oncology team.\textsuperscript{27,28} Many women are getting overtreatment for earlier stages mainly because of patient’s fear of dying cancer and doctor’s fear of cancer progressing to latest stage. These administered early-stage treatments that are more than what patients clinically need, add complexation on the health of the women as well as economic burden.\textsuperscript{28,29} Especially in stage IV, due to the spread of the cancer to several locations a more specific location focused treatment may not be enough. Treatments for this stage depend on the secondary site where the tumor cells migrated to and the tumor molecular sub-type.\textsuperscript{30,31} Overall, even if the currently existing breast healthcare advancement is showing high incidence and mortality rate, the administered treatments are not effective to significantly reduce mortality rate. In this thesis we focus on understanding the tumor microenvironment for the identification of more effective therapeutics.
1.2 Tumor Microenvironment

1.2.1 Extracellular Matrix

The extracellular matrix (ECM) is the non-cellular component of tissue and it is composed of two main compartments which are the basement membrane and the interstitial space.\textsuperscript{32,33} Both entities have their own components to complete their task. The basement membrane is a thin membrane that contains collagen IV, laminins, fibronectin, and other fundamental molecules.\textsuperscript{34} The interstitial matrix contains collagen, fibronectin, elastin, decorin, and hyaluronan.\textsuperscript{33} In general, ECM is composed of the two main macromolecules proteoglycans and fibrous proteins.\textsuperscript{35,36} This complex network in the ECM provides cells with the essential biochemical and biomechanical cues. Stromal cells produce and assemble the extracellular matrix to help in modulating the function and behavior of the cell.\textsuperscript{37} The unique and specialized components of the ECM regulate tissue integrity, cell-cell connections, interactions, and communication.\textsuperscript{38} ECM signaling in the breast tissue is tightly regulated for the development of mammary glands. If the degree of stiffness, organization, and molecular composition of ECM is altered then the cell environment may be affected as the physical assembly state of ECM is critical to cell response and function.\textsuperscript{39,40}
**Figure 5:** The extracellular matrix (ECM) is the non-cellular component of any tissue that provides the essential biochemical and biomechanical cues. ECM is composed of many proteoglycans and fibrous proteins. [Modeled after Pompili et al. (2021)]

Stromal cells are 80% of the tumor microenvironment and tumor microenvironment is 90% of the solid tumor mass.\(^1\) ECM composition in breast cancer environment facilitates in the progression of cancer. Studies have shown that the remodeling of the ECM in breast tissue highly contributes to the progression of breast cancer.\(^{41}\) Breast cancer researchers have been focusing on ECM to study the tumor microenvironment in depth to investigate the interaction between cancer cells with different ECM components. Careful analysis of the ECM protein signaling pathway involved altering the tumor microenvironment, may aid in the production of promising therapeutics.\(^{42,43}\) In the tumor microenvironment, ECM has different signatures that contain valuable information on understanding tumor progression and metastasis. The tumor microenvironment is very heterogeneous. Depending on the tumor sub-types, there are different signaling cascades that occur between the primary tumor and stromal ECM components to aid tumor progression to metastatic site.\(^{44}\) The extracellular matrix provides the cell-to-cell adhesion
for tumor cells to move by creating means for direct contact between collagen and cancer associated fibroblasts. Different breast cancer sub-types migrate to different secondary locations by employing ECM components to favor their work. As shown in Figure 6, the different signatures in the ECM are results of the cross-talk between the stromal cell components and the cancer cells in the primary location succeeding to more remodeling of ECM for cell migration (as shown in Figure 7). In this research, different ECM signatures resulted from remodeling by decidualized fibroblasts and cancer associated fibroblasts are studied.

**Figure 6:** Step by step mechanism of the remodeling of ECM created by the communicating cancer cells and tumor-associated stromal cells in the primary tumor location. The crosstalk between ECM proteins and cancer cells contributes heavily on the support that stromal cell has for cancer cells. [Adopted from Winkler et al. (2020)]

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Figure 7: A. Carcinoma in situ, tumor cells communicate with ECM components, but are restricted from migrating into the surrounding tissue. B. Tumor cells and cancer associated fibroblasts communicating via integrin for proteolytic degradation of ECM to achieve the breakage of ECM basement membrane. C. Crosstalk between the cancer associated fibroblasts and tumor cells degrades the basement membrane and reorganizes collagen fiber for tumor cell migration. [Adopted from Winkler et al. (2020)]

1.2.2 Collagen molecule assembly

One of the major components of ECM is collagen, which is the most abundant fibrous protein in connective tissue. Collagen has a tissue specific hierarchical organization (as shown in Figure 8B) consisting of three polypeptide chains, α-chains. The α-chains contain varying size of amino acids forming multiple α-chain types which then twists to form the major collagen structural component, the triple helix (its amino acid sequence domain is Gly-X-Y). Formation
of type I collagen starts with three α-peptide chains, two α-1 & one α-2, self-assembling to procollagen then the two peptide ends get removed by enzymes in the ECM to form tropocollagen, the triple helix.\textsuperscript{50} The carbon-oxygen and hydrogen-nitrogen bonds comprising the α-helix has their own dipole moment which then is amplified along the peptide chain giving the collagen molecule a permanent dipole (as shown in Figure 8A).\textsuperscript{51} The basic structural unit of collagen, the triple helix tropocollagen, group together through self-assembly into fibrils then bundle up into collagen fibers.\textsuperscript{51–54} Collagen is present in the interstitial space of the ECM along with many other fibrous proteins and it also develops to different collagen types i.e. collagen II, III, IV.

\textbf{Figure 8:} A. Collagen molecules have a permanent dipole.[Adopted from Robinson et al. (2016)]\textsuperscript{50} B. Hierarchical organization of collagen fiber.

Collagen in normal tissue reorganizes for functions such as wound healing. In breast tissue, higher collagen deposition results in dense tissue increasing risk of breast cancer.\textsuperscript{55} Tumor associated collagen signature (TACS) show significant difference in their alignment depending on their progression state in which dense collagen indicating presence of smaller tumor,
stretched fiber around the tumor boundary indicating tumor growth, and radial fiber alignment indicating possibilities for tumor invasion (Figure 9).\textsuperscript{56} Investigating the stromal cell in the complex tumor microenvironment for tumor associated collagen signatures aid in patient prognosis.\textsuperscript{17,43,57,58} Improved understanding of this environment is critical for development of better therapeutics that could possibly block the cross talk in the complex tumor microenvironment to improve breast cancer survival rate. Here in this thesis collagen signatures resulting from microstructural remodeling caused by the interaction between reactive or non-reactive stromal/tumor cells were quantified using a collagen specific imaging modality.
**Figure 9:** A. In tumor associated collagen signature -1, the dense collagen surrounding the tumor is characterized by higher intensity in signal and indicates the presence of small tumor as outlines by the yellow curve. B. In tumor associated collagen signature-2 majority of the collagen fiber are characterized by having parallel orientation to the tumor boundary. C. In tumor associated collagen signature-3 collagen fiber has irregular distribution around the tumor boundary, however, fiber orientation is mainly characterized by showing a perpendicular alignment to the tumor boundary.[Adopted from Provenzano et al. (2006)]

1.2.3 Fibroblasts

Like collagen, fibroblasts are also most common cells in connective tissue. They are major component of the ECM as they produce and reorganize ECM proteins for normal tissue
Fibroblasts engage in tissue modification and production of different ECM proteins such as collagen, proteoglycans, fibronectin, glycosaminoglycans and other glycoproteins. Fibroblasts are elongated cells that are key for collagen synthesis. They communicate with the surrounding ECM components using different mechanisms and not considered to be active remodelers of the ECM microenvironment. Myofibroblasts, here modeled using decidualized fibroblasts, are activated fibroblasts in non-cancerous stromal cell that are mainly involved in wound healing. In their activated format fibroblasts are characterized by higher α-smooth muscle actin expression and greater production ECM protein along with intense remodeling/reorganization of collagen. Due to this remodeling, there is an extensive stress in the fiber network until the location restricted wound healing or specific job is accomplished. The process of wound healing and remodeling for cancer cell progression by cancer associated fibroblasts have similar underlying mechanism.

**Figure 10**: Stromal inactive fibroblasts (a) and stromal activated (b) fibroblasts surrounded by collagen fiber and other ECM components. [Adopted from Kalluri et al. (2006)]

Cancer associated fibroblasts (CAFs) share similar characteristics as the wound healing process. However, they are different from the normal stromal activated fibroblasts such as
myofibroblasts because they are characterized by increased production of ECM protein and collagen. The tumor microenvironment is very heterogeneous in its nature and the extra ECM proteins produced by CAFs provide the cancer cell with structural signaling. Orientation of collagen fibers influences directed migration of cells by either potentiating growth factor receptor signaling or by mechanically reinforcing cell migration.\textsuperscript{67} The remodeling by CAFs could enhance metastasis as the mechanical cues between CAF and the tumor increase the stiffness of the surrounding matrix. Cancer cells communicate with their surrounding environment through different signaling cascades. The mechanoreceptors that are highly associated in the transmission of information between CAFs and cancer cells in a tumor microenvironment are integrins.\textsuperscript{68} Integrins, specifically the $\alpha$, $\beta$-integrin subunits, are non-covalently linked heterodimers that transduce signals for the communication between CAFs and cancer cells in the tumor microenvironment.\textsuperscript{68} These integrin subunits are expressed by both stromal ECM and cancer cells in which the cell-adhesion mechanical communication has strong contribution for the cancer progression to metastasis.\textsuperscript{69,70}
Figure 11: Tumor microenvironment (TME) contains cancer associated fibroblasts (CAFs) which are characterized by increased production of the ECM protein. Increased synthesis, degradation, and remodeling of fibrillar collagen in the ECM aid cancer metastasis. [Adopted from Jang et al.]^68

As studies show, tumor associated collagen signatures can serve as a biomarker for the different stages of tumor metastasis and perhaps in determining the survival rates of breast cancer patients.\(^71\) Understanding the underlying reasoning for tumor metastasis could also help in studies for potential therapeutic development to block tumor metastasis. The ability to view collagen organization in a tumor environment with sub-micron resolution to quantify collagen fiber organization is critical. Researchers have been employing several optical techniques to investigate the orientation of fibrillar collagen caused as a result of this increased remodeling in the tumor microenvironment.\(^72\)–\(^74\) In this work, two-photon excitation fluorescence microscopy for cell autofluorescence imaging and second harmonic generation microscopy for collagen fiber imaging are discussed as an outstanding imaging modality for tumor microenvironment study.
1.3 Instrumentation

The microscope is a ubiquitous instrument used in biomedical science and engineering. Optical instruments could be designed in several different ways to fit the appropriate task. The history of optical instruments, mainly microscopes, dates back to the 16\textsuperscript{th} century but, it was first introduced to biologists after Robert Hooke viewed cells.\textsuperscript{75,76} The development of microscope started from a simple microscope comprised of a single lens. Since then, microscope underwent a significant modification to a more complex design so that optimized resolution is achieved, and aberration is minimized. The development of modern microscope has accelerated the study of life science.

Many researchers use different microscopy techniques to investigate cell extracellular matrix (ECM) such as wide-field fluorescence microscopy, confocal microscopy, and nonlinear microscopy. In recent years nonlinear laser scanning microscopy is highly selected to study the ECM over the other microscopy techniques due to its advantages for deeper penetration in thicker samples, less photobleaching, intrinsic optical sectioning, higher spatial resolution, and ability to collect endogenous information.\textsuperscript{77,78} Nonlinear laser scanning microscopy significantly enhanced the study of collagen structure in disease and normal states. The two nonlinear optics that are highly utilized to study the organization of collagen fiber in cancerous environments are two-photon microscopy and second harmonic generation microscopy.\textsuperscript{79,80,81} Both optical techniques have their own mechanism of revealing the details in the unseen microscopic phenomenon. The fundamental parts for the light pathway from the source to sample then to a detector for both these techniques is similar except the emission filter. In this work, the specific components of these nonlinear optical techniques in their application to study TACS is explained.
1.3.1 Instruments used in Collagen imaging

1.3.1.1 Fluorescence Microscopy in ECM and collagen imaging

In the 1800s and early 1900s, the phenomenon of fluorescence was highly investigated. Alexander Jablonski graphically described the energy states of fluorescence in what is known today as a Jablonski diagram. The graphical description of the absorption and emission patterns of light in the Jablonski diagram advanced the knowledge of fluorescence. Then later the invention of lasers in the 1960s advanced the development of fluorescence microscopes and set the stage for the invention of laser scanning microscopes. Soon after Carl Zeiss introduced the first laser scanning fluorescence microscopy in 1982. The production of pulsed lasers advanced the development of nonlinear microscope techniques such as the two-photon fluorescence microscopy and second harmonic generation microscopy. The advancement in the production of lasers, lenses, objectives, mirrors, filters, and detection methods in the later years enabled researchers to design and build homemade multiphoton microscopy techniques.

1.3.1.2 Two photon excitation fluorescence microscopy

The first theory of the two-photon process was made by Gopper Mayer with the first experimental observation of the process in 1961. The introduction of pulsed lasers was a great contribution for the advanced development of the multiphoton microscopy system. Since then, multiphoton laser scanning microscopy (MLSM) has become a useful field under the nonlinear optical microscopy for biological studies.

The two-photon process, unlike a single-photon process, requires two photons to simultaneously excite a molecule from its ground state. In a single-photon process a shorter wavelength is mostly used to provide sufficient energy for the molecules, however, in a two-photon process a longer wavelength is used. There are several advantages to using a longer
wavelength in the two-photon excitation process such as deeper penetration in thicker samples, minimal photodamage, high spatial resolution, ability to deeply investigate components in highly scattering biological sample. These advantages come from the fact that energy and wavelength are inversely related.

Figure 12: Jablonski diagram of one-photon (A) and two-photon (B) fluorescence processes. In one photon fluorescence only one photon of shorter wavelength is absorbed by fluorophores to get to excited state from the ground state. In two-photon fluorescence, a longer wavelength is used to excite fluorophores and unlike one photon here two photons are simultaneously being absorbed by fluorophores. [Adopted from So, P.T et al (2001)]

The underlying physics of two-photon excitation fluorescence microscopy (TPEF) rely on the fluorophore's ability for a two-photon absorption cross-section. The two-photon absorption cross-section is the measure for the probability of the absorption process by intrinsic
or exogenous fluorophores. Illumination process in two-photon process is pulsed in which the molecule’s ability for simultaneous absorption of two photons of half the excitation wavelength double the frequency at a reduced energy is critical (see Figure 13). Since two-photon fluorescence is a nonlinear process, the intensity of the emission signal is the square of the irradiating signal. As shown in the Jablonski diagram in Figure 13, two-photons at a given frequency and wavelength get absorbed by a fluorophore to produce one photon of less than twice the frequency and less than half of the incident photons.

![Two-Photon Excited Fluorescence](image)

**Figure 13**: Two-Photon Excitation Fluorescence Jablonski Diagram. [Adopted from Masters 2010]^{82}

In 2PEF microscopy the key components are a mode locked femtosecond pulsed laser (mostly Titanium-Sapphire laser), an objective lens, dichroic mirror, bandpass filter, and photon counting head.^{88} During objective lens selection the important specifications to consider are the magnification, the numerical aperture, the working distance, and its efficiency in excitation signal collection. When choosing a collection optics, it is important to consider their collection efficiency. Quantum efficiency, response time, wavelength sensitivity, and linearity are
important things to balance on a PMT. The considerations needed to take when selecting laser are the pulse width, peak power, repetition rate, and wavelength tunability range.\(^{89}\)

### 1.3.1.3 Second Harmonic Generation Microscopy (SHG)

Second Harmonic generation (SHG) has been around since 1974 after the introduction of pulsed lasers but for the last two decade SHG has been highly utilized in biological research.\(^ {80}\) SHG is also a nonlinear optical and the Jablonski diagram below shows the SHG process. SHG was introduced as the nonlinear biological sample imaging technique in 1986 after its first publication as a nonlinear technique in 1978.\(^ {90}\) In SHG there is no absorption and emission rather signals are generated from the intrinsic property of the molecules in the biological sample which have non-centrosymmetric order. The lack of symmetry in the assembly of collagen molecules makes it compatible with the physics of SHG signal generation. SHG as a nonlinear process also permits deeper penetration in thick sample imaging with intrinsic optical sectioning. Furthermore, in SHG there is no photobleaching and phototoxicity since it is mostly based on scattering rather than absorption.

SHG is also a coherent process that is dependent on the dipole organization of the molecules in the order of the SHG wavelength. SHG has directionality in which a specific signal pattern comprises the forward and backward propagating components. The characterization of the emitted signal for SHG is determined by the phase matching of signals emitted from individual molecules. SHG contrast is dependent on the orientation of the molecules in the microstructures in the direction of the laser propagation and the polarization of the incident beam. Mathematically the relationship between the polarization, the ability of the molecule to generate second harmonic signals (linear susceptibility), and the electric field is defined as

\[
P(t) = X^{(2)} E^2(t)
\]
where \( P(t) \) is the polarization at time \( t \) resulting from the dipole moment of the molecules within a certain volume, \( X^{(2)} \) is the second order susceptibility tensor, and \( E \) is the electric field.

**Figure 14:** Second Harmonic Generation Jablonski diagram (Left) showing two photons of half the frequency twice the wavelength interacting with a non-centrosymmetric medium to produce one photon of double the frequency but half the wavelength of the incoming photon. The fundamental of SHG is its directionality which is dependent on the dipole organization of collagen molecules in the order of \( \lambda_{\text{SHG}} \) within the optically nonlinear medium (Right). [Adopted from Mostaco-Guidolin, L. et al (2017)]

The fundamentals of SHG are its directionality and phase matching which is used to characterize the different collagen architecture. Phase matching of the half frequency and double
frequency in SHG must be in phase so that they do not destructively interfere. In an ideal non-centrosymmetric medium, SHG signals are strongly forward directed and mostly remain in phase. However, in biological structures such as collagen, it has been known that there are both forward and backward signals.\textsuperscript{91} SHG signals in collagen imaging arise from the dipole moment in the alpha helix. Fibrillar assembly ordered at the size scale of SHG wavelength are mainly forward directed. However, the inherent properties of collagen molecule organization does not result perfect phase matching. The presence of the difference in order, size, and shape present in collagen molecules in disease and normal states contributes to the signals phase-matching.\textsuperscript{92–94} For this reason, SHG is anisotropic. The directionality effect of SHG can be quantified by calculating the forward propagation by the backward propagation (\( F_{\text{SHG}}/B_{\text{SHG}} \)) and the phase-matching consideration can mathematically be expressed as follows.

\[
k = \frac{2\pi}{\lambda}
\]

\[
\Delta k = k_{2\omega} - 2k_\omega
\]

I\textit{edally} \( \Delta k = 0 \) but in collagen \( \Delta k \neq 0 \)

\[
\text{Coherence length} = \frac{2\pi}{\Delta k}
\]

The physical property of SHG that is needed to answer the desired question is its phase matching. Unlike 2PEF signals SHG signals are produced from the interaction between the incoming photon with the non-centrosymmetric medium instead of molecules absorbing and emitting signal. The presence of the difference in order, size, and shape present in collagen molecules in disease and normal states contributes to the signals phase-matching. The signals are
produced without the presence of any exogenous material which gives intrinsic information of the collagen molecule assembly.

SHG signals are produced strongly in the range of 800nm to 890nm as seen in most biological research.\textsuperscript{95,96} In this work the mode locked Ti-Sapphire pulsed laser was used. This is a pulsed laser unlike a continuous laser it emits light at a repetition rate which describes the frequency in which pulses are emitted. The use of pulsed laser accommodates the probability for the simultaneous arrival of the smaller energy photons at the sample for signal generation.\textsuperscript{97} The time measured across the pulses is known as the pulse duration in which shorter pulse duration with high peak power deliver minimal heat to the sample also decreasing photodamage on the sample. The pulse width, a measure of the time between the beginning and end of the pulse, affects the average power in a directly antagonist manner for a given period. The period describing the time between the start of one pulse and the start of the next pulse, relates to the average power inversely. Therefore, increasing pulse width of the laser beam while decreasing its period, increases the average power. Overall, a smaller pulse duration with a higher average peak power is a preferred feature for the nonlinear process and deeper penetration in thick and highly scattering samples.\textsuperscript{88}

In the excitation pathway, the important components to consider are the half wavelength and quarter wavelength waveplates, the scanning head, dichroic mirror, and the objective. The half wavelength ($\lambda/2$) waveplate holds the polarization of light by a half wavelength and changes its linear polarization direction. The quarter wavelength ($\lambda/4$) waveplate holds the polarization light by a quarter of the wavelength and converts it from linear to circular polarization. The scanning head, to be used along with an upright microscope, has two channels that is used for both the forward and backward signal emission. The dichroic mirror used on the way of the laser
beam before it gets to the objective is selected based on its higher transmission efficiency for the near IR wavelength and higher reflection efficiency for the SHG wavelength. An objective with features such as water immersion, high Numerical aperture (NA), low magnification, and longer working distance along with a better transmission efficiency of the near-IR wavelength is selected for the design. Selecting a high NA low magnification water immersion objective corrects spherical aberration and it is useful for a higher resolution.

The forward and backward emission pathway must contain the components dichroic mirror, lenses, SHG filter, a Photo-multiplier tube, and detection mechanism. The dichroic mirrors are selected based on their ability to reflect emitted light at the SHG wavelength to the detection pathway. The lenses on the way of the emission focuses the signal that goes to the SHG bandpass filter. From the bandpass filter the signal goes to a photon counting head which couples a photomultiplier tube (PMT) with a built-in optimization system. As the received photon signal goes through the PMT it gets changed to a current with a higher electron gain. The output measurement done in a photon count mode gives a better signal to noise ratio. Details of the specific parts used in the design are further explained in chapter 2.

1.3.1.4 SHG competitive ECM imaging modalities for clinical use

PolScope is an imaging alternative for collagen imaging that can be used in place of SHG and regular staining. PolScope unlike the regular polarization microscope uses a Liquid Crystal (LC) compensator that eliminates the physical moving of specimens to find birefringence in the sample. Collagen being a highly birefringent component of the ECM (although there are other birefringent in the ECM collagen is highly dominant), it is suitable for the LC-PolScope imaging. The orientation and alignment of collagen in a smaller sample can be studied using the PolScope without requiring any staining and any higher optical complexation for any pathological studies.
Studies have also shown that quantitative assessment of collagen alignment and orientation completed on images taken by both LC-PolScope and SHG show a higher correlation. The advantage of LC-PolScope over a traditional Polarization microscope lies in the fact that any orientation scale in the fibrillar organization of collagen molecules could be demonstrated and the liquid crystals used as compensators are controlled electronically to produce any polarized light. Therefore, using PolScope could be a simple and effective label free alternative collagen imaging modality to implement in clinical and any pathological studies for investigation of cancerous and non-cancerous tissue samples.⁷²

In addition to the label free SHG and 2PEF microscopy, there also exists staining techniques that give even more detailed information about the organization of collagen fiber. The staining technique that is highly in use for collagen fiber imaging is the picrosirius red (PSR) stain which specifically highlight collagen fiber. PSR stained sample can then be imaged best using a fluorescence microscope to give information on the orientation and organization of collagen locally in a specific region of interest. This is a robust and cheaper technique compared to the multiphoton laser scanning microscopy (MPLSM) that could readily be used for any pathological studies. However, this labeling technique fails in relying information on how collagen fiber is influencing changes to the cell environment or how it is being influenced by the surrounding tumor and normal cells in diseased and normal conditions. Furthermore, although the staining technique specifically binds to collagen and allows for local quantitative analysis, the fact that the technique does not allow any analysis to be done in the ECM surrounding the collagen fiber makes its application limited.⁷³

Another label-free imaging modality that can potentially be used for fibrillar collagen in cancerous environments is Polarimetry. Polarimetry is a technique that uses polarized light to measure the different organizational compositions of collagen and other components in
tissue in diseased and normal conditions. This imaging method could easily be incorporated in a traditional microscope used by pathologists to look into samples. Unlike highlight advanced imaging modalities such as SHG, polarimetry provides a cheaper and easily applicable label free solution to any tumor microenvironment investigation. However, the limitation of this methodology is that it is a little time consuming, has a lower resolution, and less sensitive to collagen organization. Polarimetry is not collagen specific, it might not be quite suitable for collagen specific quantification.\textsuperscript{74}
CHAPTER 2
MATERIALS AND METHODS

2.1 Methods

2.1.1 Experimental Planning

A total of six low density collagen gel (2 mg/mL) samples, two control and four experimental (co-cultured breast cancer cell and fibroblast cell lines) were prepared. These collagen gels are non-scattering which is useful to get F/B directly from the fiber orientation. The positive control gels contained only decidualized fibroblasts (dBJ) activated by the secondary messenger cyclic adenosine monophosphate (cAMP); whereas the negative control gels were seeded with normal fibroblasts (BJ). The fibroblast used here is BJ which is a normal human foreskin fibroblast taken from a neonatal male. Co-cultures of normal fibroblasts and cancer cells model a non-reactive stroma where co-cultures of activated fibroblasts and cancer cells model a reactive stroma. In normal stroma, ECM protein production is tightly controlled; however reactive stroma ECM protein production is dysregulated leading to significant cell-mediated collagen synthesis and remodeling. To model normal and abnormal stromal fibroblasts, we cultured normal and cAMP activated fibroblasts respectively.

In the experimental groups, two types of cancer cell lines were seeded separately in the cAMP activated fibroblasts containing collagen gel and normal fibroblast containing collagen gel. The first experimental samples contained dBJ and the MCF7A cancer cell line whereas the other sample contained the BJ with MCF7A cancer cell lines. MCF7A cancer cells are hormone responding breast cancer cell lines with human epidermal growth factor 2 (HER2), estrogen (ER), and progesterone (PR) receptors. They retain ideal characteristics particular to mammary epithelium such as processing of estrogen via estrogen receptors in the cell cytoplasm, however, it is a relatively less aggressive breast cancer cell line. In the gel model MCF7A_BJ
and MCF7A_dBJ, MCF7A cancer cell lines were cultured with normal fibroblasts and cAMP activated fibroblast to model non-reactive reactive stroma respectively. In the last two experimental gel models MDA_MB_231_BJ and MDA_MB_231_dBJ, the cancer cell line MDA_MB_231 were cultured with normal fibroblasts and cAMP activated fibroblast to model non-reactive reactive stroma respectively. MDA_MB_231 cancer cells are ER, PR, and E-cadherin negative receptors but, they lack the growth factor receptor HER2 which makes it a triple negative breast cancer cell sub-type. MDA_MB_231 are very aggressive cancer cell lines that often are difficult to treat in clinical diagnosis.

In gels where the cAMP activated fibroblast (dBJ) is co-cultured with either of the tumor cells, a reactive stromal fibroblast that is more like myo-fibroblasts, interacts with the two different cancer cells. This demonstrates if the impact of reactive stromal fibroblast on collagen remodeling is dependent on the tumor cell subtype. In gels where normal fibroblast is co-cultured with either of the tumor cells, a non-reactive stromal fibroblast interacts with the two different cancer cells. These models, therefore, demonstrate the collaborative impact that cancer associated fibroblasts and cancer cells have on the remodeling of collagen fiber in a tumor microenvironment. Moreover, the models also demonstrate the difference in the crosstalk between cancer associated fibroblasts and cancer cells when they associate with different tumor cell subtypes.

After 72 hour all the samples were fixed with 4% paraformaldehyde (PFA) for Second Harmonic Generation forward and backward imaging. The sample was also labeled with Cell Tracker to ease the process of tracking the cell interactions.
Figure 15: In vitro collagen gel model of normal healthy stromal deactivated fibroblasts – BJ (A), normal healthy stromal activated fibroblasts – dBj (B), non-decialualized fibroblasts co-cultured with MCF7A (C) and MDA_MB_231 (C) cancer cell lines, and decidualized fibroblasts co-cultured with MCF7A (E) and MDA_MB_231 (F) cancer cell lines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ</td>
<td>(-) Control</td>
<td>Sample contains non-decialualized fibroblasts that are stromal deactivated fibroblasts.</td>
</tr>
<tr>
<td>dBj</td>
<td>(+) Control</td>
<td>Sample contains decidualized fibroblasts that are stromal activated fibroblasts.</td>
</tr>
<tr>
<td>MCF7A_BJ</td>
<td>Experimental</td>
<td>Sample contains non-decialualized fibroblasts co-cultured with ER+ cancer cell lines.</td>
</tr>
<tr>
<td>MCF7A_dBJ</td>
<td>Experimental</td>
<td>Sample contains decidualized fibroblasts co-cultured with ER+ cancer cell lines.</td>
</tr>
<tr>
<td>MDA_MB_231_BJ</td>
<td>Experimental</td>
<td>Sample contains non-decialualized fibroblasts co-cultured with triple negative cancer cell lines.</td>
</tr>
<tr>
<td>MDA_MB_231_dBJ</td>
<td>Experimental</td>
<td>Sample contains decidualized fibroblasts co-cultured with triple negative cancer cell lines.</td>
</tr>
</tbody>
</table>
2.1.2 Image Acquisition

The collagen gel samples were imaged using the multiphoton laser scanning techniques SHG and 2PEF microscopy. The 2PEF microscopy was used to take the cell fluorescence signal and the SHG was used to get the intrinsic forward and backward SHG signals from the collagen network. The Titanium-Sapphire laser was tuned at 750 nm for the cell imaging. A 582/64 bandpass filter was used to epi-detect Cell Tracker fluorescence signals. The laser was tuned at 890 nm for collagen imaging in which a 448/20 bandpass filter was used for the backward emission detection path and simultaneously the forward SHG signal is collected.

Figure 16: Tilbury lab home built two-photon excitation fluorescence and second harmonic generation microscope set-up. [Adopted from William Patrick Breeding]¹⁰²

2.1.2.1 Photon Budget Calculation and Sensitivity Analysis

A photon budget analysis describing the amount of light needed to generate SHG and 2PEF signals is described here. Both optical processes are nonlinear meaning that the intensity of the emission signals is proportional to the square of the radiation signal. In the analysis the
number of excitation photons, the number of emission photon, and signal to noise ratio are the important considerations. The overall efficiency of the system in achieving optimum values for these three main things is determined by the collection efficiency of the objective lens, the light transmission & reflection efficiency of other optical components (both in the excitation and emission path), and quantum efficiency of the detector.

The excitation path is used to estimate the power of the light source and the emission path is used to estimate the signal to noise ratio. In the excitation path the laser (light source), the half and quarter waveplates, the dichroic mirror, and the objective lens are considered to calculate the number of excitation photon (see Figure 1A). The light source for both SHG and 2PEF is a Ti-Sapphire laser. A pulsed light source for the nonlinear optical system. In the emission path the objective lens, the dichroic mirror, the lens, and the emission filter are considered to calculate the number of emitted photons. Here, a water immersion objective with numerical aperture of 0.8 is used for both the excitation photon path and emission photon path. Therefore, the volume in which the excitation photons go through, and the emission photons leave can be assumed to be the same.
Figure 17: Considerations in the excitation pathway (A) to estimate the excitation number of photon and consideration in the emission pathway (B) to estimate the emitted number of photons from the sample.
The specification of each component is:

➢ Olympus LUMPLFLN 40XW Objective
  • Transmission efficiency = 80%
  • Numerical aperture = 0.8

➢ 448/20 bandpass filter
  • Transmission efficiency for wavelength between 438-458nm is 98%

➢ Dichroic mirror
  • Transmission efficiency = 93%
  • Reflection efficiency = 98%

➢ Considering the bandpass filter, DCM, and objective ~76.83% of the emitted photons reach the detector.

The focal volume (FV) describing the area in the sample where the photon concentration is higher can be obtained by considering the lateral ($\omega_{\text{lateral}}$) and the axial ($\omega_{\text{axial}}$) points spread functions. The ideal point spread function is the three-dimensional diffraction pattern of light emitted from the laser source in the specimen and transmitted to the image plane through the objective’s numerical aperture. Both the lateral and axial points spread functions are wavelength dependent and they can be calculated as follows. The lateral point spread function focuses on the diffraction pattern of the emitted light in the xy direction as it passes through the back aperture of the objective lens resulting a 3D interference. The axial point spread function focuses on the diffraction and interference in the xz direction.
\[
\omega(z) = \omega_o \sqrt{1 + \left(\frac{z}{z_R}\right)^2}
\]

\[
z_R = \frac{b}{2} \left( \frac{\lambda}{\pi \omega_o} \right) R(z) = z \left[ 1 + \left(\frac{z_R}{z}\right)^2 \right]
\]

\(\omega(z)\) = Half Beam Diameter

\(\omega_o\) = Beam Waist

\(z_R\) = Rayleigh Range

\(z\) = Axial Distance

\(\theta\) = Half Angle Divergence

\(b\) = Confocal Parameter

\(R(z)\) = Radius of Curvature

\(\lambda\) = Wavelength

\(\omega_R(b/2)\) = Rayleigh Half Diameter
Focal Volume (FV)

Lateral point spread function for NA > 0.7

\[ \omega_{\text{lateral}} = \frac{0.325 \lambda}{\sqrt{2} NA} = 255.66 \text{nm} \]

\[ \omega_{\text{axial}} = \frac{0.532 \lambda}{\sqrt{2} \left( \frac{1}{n^2 - \sqrt{n^2 - NA^2}} \right)} = 473.62 \text{nm} \]

\[ \text{FV} = \pi^2 \left( \omega_{\text{lateral}} \right)^2 \left( \omega_{\text{axial}} \right) = 1.724 \times 10^8 \text{nm}^3 \]

The number of photons coming from the light source can be obtained by considering the number of excitation photon, the reflection efficiency of the dichroic mirror, the transmission efficiency of the excitation filter, and the transmission efficiency of the lenses in the excitation path. The number of excitation photon per second required for illumination of the entire field of view can be characterized by the photon flux. First the number of incoming photons from the laser are obtained from the total energy of the light source. Then using the power at which the laser was set to collect the images from the sample, the number of photons per second from the source can be obtained. Then to get the photon flux first the beam waist of the laser needs to be calculated from the focal length (f), the excitation wavelength (\( \lambda \)), and the beam radius (d) of the laser. Then the amount of power from the light source in the entire field of view can be estimated by the photon density using the photon flux and the energy of the photons.

Energy

\[ E = \frac{hc}{\lambda} = \frac{(6.626 \times 10^{-34} \text{Js})(3 \times 10^8 \text{m/s})}{8.90 \times 10^{-7}} = 2.234 \times 10^{-19} \text{ J/photon} \]
➢ **Power (W)**

\[ W = \text{Number of Photon} \times E \]

Number of Photon = \( \frac{W}{E} = \frac{0.318 \text{J}}{2.234 \times 10^{-19} \text{J}} \)

Number of Photon = \( 1.42 \times 10^{17} \frac{\text{photons}}{\text{sec}} \)

➢ **Photon flux**

**Beam waist:** \( \omega_o = \frac{2\lambda f}{\pi d} = \frac{2(890 \text{nm})(4.5 \times 10^6 \text{nm})}{\pi(2.1 \times 10^7 \text{nm})} = 121.41 \text{nm} \)

**Photon flux:** \( \phi = \frac{\text{Number of photons}}{\text{Area (cm}^2\text{)}} \)

\( \phi = \frac{\text{Number of photons}}{\pi (\omega_o)^2 \text{cm}^2} \)

\( \phi = \frac{(1.42 \times 10^{17}) \text{photons}}{\pi (1.2141 \times 10^{-5})^2 \text{cm}^2} \)

**Photon flux:** \( \phi = 3.066 \times 10^{26} \frac{\text{photons}}{\text{sec cm}^2} = 3.066 \times 10^{30} \frac{\text{photons}}{\text{sec m}^2} \)

The calculated photon flux describing the number of photons striking in the area per second is greater. But for the wavelength the laser is tuned at, the energy content of each photon is proportional. There is no absorption of photons and loss of energy in SHG rather the emitted photons have twice the energy of the excitation photons. Assuming there is no photon
destruction and considering the bandpass filter, the dichroic mirror, and the objective lens in the emission pathway, the number of photons leaving the volume can be calculated as shown below

➢ **Power Density (Pd)**

\[
Pd = \Phi \frac{hc}{\lambda} = \Phi E
\]

\[
Pd = \left(3.066 \times 10^{27} \text{photons/sec}^2\right) \times (2.234 \times 10^{-19})
\]

\[
Pd = 6.849 \times 10^{12} \text{W/cm}^2 = 6.849 \times 10^{16} \text{W/m}^2
\]

➢ **The number of excitation photon entering:**

\[
n_{0,\text{source}} = \frac{n_o}{R_D \tau_{\text{EF}} \tau_{\text{obj}}}
\]

\[n_{0,\text{source}} = \text{number of photons from the light source}
\]

\[n_o = \text{number of photons entering a volume}
\]

\[R_D = \text{reflection efficiency of the dichroic mirror}
\]

\[\tau_{\text{EF}} = \text{transmission efficiency of excitation filter}
\]

\[\tau_{\text{obj}} = \text{transmission efficiency of the objective}
\]

\[
n_o = \left(R_D \tau_{\text{EF}} \tau_{\text{obj}}\right) x (n_{0,\text{source}})
\]

\[
n_o = (0.98 \times 0.93 \times 0.80) \times (1.42 \times 10^{18}) = 1.035 \times 10^{18} \frac{\text{photons}}{\text{sec}}
\]

➢ **Considering the bandpass filter, dichroic mirror, and objective 76.83% of the photon reaches the detector**

\[
n_d = 0.7683 n_o
\]
\[ n_d = 0.7683 \times (1.035 \times 10^{18}) \text{photons sec}^{-1} \]

\[ n_d = 7.952 \times 10^{17} \text{photons sec}^{-1} \]

➢ **Photon counting head: Count sensitivity**

\[
\text{Count Sensitivity} = \frac{\text{Number of counted pulses (output pulse)}}{\text{Number of incident photons}}
\]

\[
\text{Detection efficiency} = \frac{N_d}{N_p}
\]

Detection efficiency = Quantum efficiency (\(\eta\)) x Collection efficiency (\(\alpha\))

\[
\text{Count Sensitivity (Detection efficiency)} = 720382 \text{s}^{-1} \text{pW}^{-1}
\]

### 2.1.2.2 Sensitivity Analysis

Points to consider for sensitivity analysis

➢ **Size:** - determines the voxel size with excitation wavelength of 890 nm.

\[
V = (\Delta X)(\Delta Y)(\Delta Z)
\]

\[
\Delta Z = \frac{\lambda_{ex}}{2NA^2}
\]

\[
\Delta Z = \frac{0.00089m}{2(0.8)^2} = 0.00070 \text{ m}
\]

➢ **Intensity:** - the signal intensity that is a function of energy flux expressed as energy per area per time.

\[
I_0 = \frac{E}{(A)(t)}
\]
The intensity can also be represented in photons per cm$^2$ s equal to the photon flux $3.066 \times 10^{26}$ photons cm$^{-2}$ s$^{-1}$.

- **Spatial resolution**: this is the ability of the microscope to differentiate neighboring structures as separate. The axial and lateral resolutions can be calculated by considering the refractive index of water, wavelength, and numerical aperture of the objective.

  \[
  \text{Axial Resolution} = \Delta z = \frac{1.22\lambda}{2\sqrt{2\text{NA}}} = \frac{1.22(890\text{nm})}{2\sqrt{2}(0.8)} \approx 480\text{nm}
  \]

  \[
  \text{Lateral Resolution} = \Delta \rho = \frac{1.5n\lambda}{\text{NA}^2} = \frac{1.5(1.33)(890\text{nm})}{(0.8)^2} \approx 2,774\text{nm}
  \]

- The effective area for the photon count head is calculated using the circle $A = \pi r^2$; it has a diameter of 5 mm.

- The pixel size that corresponded to the image pixel is the biggest square that could possibly fit as shown in the figure below.

  The size of the square is

  \[
  a^2 + a^2 = D^2
  \]

  \[
  2a^2 = D^2
  \]

  \[
  a^2 = \frac{(5\text{mm})^2}{2}
  \]

  \[
  a = 3.54 \text{ mm} = 13.38 \text{ pixel}
  \]

  \[
  \text{Voxel} = V = (\Delta X)(\Delta Y)(\Delta Z) = (3.54\text{mm})(3.54\text{mm})(3.54\text{mm}) = 44.362\text{mm}^3
  \]
➢ The images produced were 512 x 512 pixel and 180 microns for the entire field of illumination.

![Diagram showing pixel size and micron size comparison]

➢ The photon capture efficiency of the first lens

\[ \gamma = \left[ 1 - \sqrt{1 - \left( \frac{NA}{n} \right)^2} \right] \]

\[ \gamma = \left[ 1 - \sqrt{1 - \left( \frac{0.80}{1.33} \right)^2} \right] = 0.10056 \]

The calculations above indicate that the exposure of the samples to the calculated photon flux indicates the maximum photo bleachability of the sample if there was absorption. The proportional relationship between intensity and photon flux here in the above calculation indicates that the selected laser power for the SHG wavelength gives a reasonable intensity within the region of interest. Since signals in SHG are generated without absorption, the variable that would affect the sensitivity of the system if changed is the power. If the power is increased to a higher amount, then some areas in the image would be saturated. Since the \( F_{SHG}/B_{SHG} \) ratio is calculated based on the pixel value, saturated areas could affect the resulting value.
2.2 Image Analysis

After the image acquisition, the processing of images of the biological samples is needed to extract the necessary information. Image processing can be defined in many ways depending on the specific research question. In this work, image processing is focused on cell segmentation for pixel based localized FSHG/BSHG ratio analysis. Image processing for cell segmentation can be either manual or automated depending on factors such as number of images that needed to be analyzed, time, speed, accuracy, and consistency. Manual image processing is useful when identifying a ground truth but, it could be very time consuming and maintaining consistency in the image processing might be a difficult task to achieve. However, the ground truth obtained from manual image processing is a great input when programming for automated image processing.

Image segmentation is one type of biological image processing. In image segmentation, one image is partitioned into several regions for a specific analysis purpose i.e., further particle analysis. Biological image segmentation, in particular cell segmentation, can be achieved with either intensity-based segmentation or other technique. The ultimate goal of segmentation is to separately extract the needed foreground region from the background. However, depending on the cell image type there are challenges in cell segmentation. In this work cell segmentation of the fluorescent cell images is challenged by intensity based segmentation. The cell fluorescence image is difficult to segment based on intensity alone because of the higher contrast from the artifacts that appear on the images. To perform effective cell segmentation additional pixel by pixel segmentation approach is required. Pixel by pixel segmentation is a technique in which pixels representing particular cell features are selected by highlighting regions and classified as
one class. Moreover, the pixel-by-pixel segmentation makes the partitioning of the cell regions easier without needing a rigorous thresholding step.

The outcome from the proper segmentation of the cells is critical to obtain a localized $F_{\text{SHG}}/B_{\text{SHG}}$ ratio analysis. The $F_{\text{SHG}}/B_{\text{SHG}}$ ratio obtained from the bulk image analysis does not give detail information to answer the research question stated above in this chapter. In bulk analysis, the alterations on the collagen fiber microstructure are considered from the overall region of the image whereas in localized analysis the cell induced changes can closely be assessed. Here, the cell localized analysis is used to look for the distinct collagen remodeling signatures resulting from the different stromal/tumor cell interactions that are modeled in the in vitro gels. The specific cell segmentation pipeline described below is developed to answer the question; are there distinct collagen remodeling signatures induced from the interactions between decidualized fibroblast with tumor cells, and the interaction between normal fibroblast with tumor cells that contribute to metastasis?

### 2.2.1 iLastik Cell Segmentation

The cell segmentation process was carried out using the software iLastik which is a machine learning based image analysis software. iLastik software is a deep learning package based on convolutional neural networks enabling pixel and object level classification. The neural network is trained for the pixel-by-pixel semantic segmentation followed by object classification. Semantic segmentation here refers to the grouping of the cell features, other artifacts, and the background to the same pixel class. Differentiation of the foreground and the background classes was first completed manually using selected images which then were fed to the network as ground truth. Then batch processing can be completed for all the images. Prior to
the image segmentation, cell image pre-processing was completed to generate *Max Z-projection* of cells from the optical sections containing the same cell. This projection considers the maximum pixel intensity in every optical section to produce one image. The projection is selected because the produced image shows all the cell features presented in every slice (as shown in Figure 18) compared to average and minimum intensity projections. This was a very applicable approach for this work because in the output image the lower and higher intensity of certain cell feature in the optical sections is balanced. In optical section where cells have higher intensity, semantic segmentations can easily be performed but those optical sections with lower intensity are quite challenging even for pixel-wise segmentation. Additionally, during batch processing, cell features with low intensity mistakenly get classified as part of the background class rather than the foreground class. Since the aim of the cell segmentation is to create cell mask for the depth independent \( F_{SHG}/B_{SHG} \) analysis, taking this max projection would make sure that every cell feature is considered. Furthermore, the localized \( F_{SHG}/B_{SHG} \) analysis is depth independent and only considers pixel value of the collagen forward and backward images so the pixel values from the cell fluorescence does not affect the final result.
Figure 18: Maximum intensity Z-projection of 2PEF cell images prepared to be used as an input for cell segmentation.

In iLastik there are several workflows but the two selected workflows in this work are Pixel-Classification and Object-Classification. Pixel-Classification workflow produces semantic segmentation of images in which the user defines each class by selecting the different desired number of classes using different colors (Figure 19B). iLastik then gives the probability map by estimating every pixel in the image based on the pixel values in the user labeled class (Figure 19C). However, the probability map only separates the foreground and the background based on the pixel annotation which does not include instance segmentation. Instance segmentation is a process in which every object in an image is detected, segmented, and classified. In instance segmentation different object classes are formed where each cell is treated as a single entity to be classified into different populations. The pixel wise semantic segmentation fails in distinguishing between classes of different object characteristics because it treats all the cells in the optical section as a single entity. As the cells in one optical section are in different size and shape, the probability map from the pixel segmentation needs to be further segmented out with object
classification. This is also beneficial for later batch processing in object classification. In object classification, first, the imported probability map is smoothed with a Gaussian filter of sigma 5.0 (this can be selected in the feature map section) and thresholded. The different object classes could then be selected to train the network by simply color coding the different objects. The output object prediction image is then exported to ImageJ for further analysis (see Figure 19D).

**Figure 19**: Process flow diagram (A-E) for semantic segmentation of cells using pixel + object classification.
2.2.2 F/B Ratio Analysis on Image J

Segmented cell images imported from iLastik were loaded on ImageJ for the next image analysis process to obtain the localized F_{SHG}/B_{SHG} ratio (see Figure 20A). First, the imported image, which has foreground 1-pixel and background 2-pixel range, was converted into a binary image of foreground pixel 0 and background 255 pixel (Figure 20C). The main advantage in converting all iLastik imported images to binary image is to allow easy manipulation of the cell regions for the desired analysis without needing to worry about Thresholding. All the binary images were converted to mask which converts the images into black (background) and white (foreground) based on the threshold setting in the images. The masks were then added to ROI manager to count the individual cells for a per cell base localized analysis. Before further analysis was performed the cell masks were dilated by 10 pixel (3.5 µm) for the localized analysis (Figure 20B). Once the cells in the original mask and the dilated mask were counted the images were converted to a 32-bit image. The 32-bit masks (original and dilated) were processed with image math to change the background pixel to not a number (NAN) (Figure 20D-E). This way the result table represents solely the F_{SHG}/B_{SHG} in each ROI without needing to worry about the background pixel value from the cell mask being considered in the image math.
**Figure 20:** Fiji image analysis process (A) preparing cell ring ROIs (B-E) for localized F/B ratio calculation.

The collagen images from the forward emission and the backward emission were analyzed separately (see Figure 21) from the cell segmentation until the final image multiplication step. First, the forward (Figure 21A) and backward (Figure 21A’) image stacks corresponding to the cell stack max Z-projection were loaded on ImageJ. The forward (Figure 21B) and the backward (Figure 21B’) stacks were Thresholded with Li thresholding algorithm separately to filter out noise before dividing the stacks (Figure 21C & 21C’). The resulting image (Figure 21D) from the image division was then multiplied with the cell ROI image. Refer to the Appendix for the FIJI macro.
Figure 21: Image operation process for the forward and backward SHG images division. Image math process presenting the multiplication between the F_{SHG}/B_{SHG} ratio for the cell localized analysis.
2.2.3 F/B Ratio Statistical Analysis on R

In Excel, the average $F_{SHG}/B_{SHG}$ ratio of every cell was calculated from the number of slices in which the cell max z-projection was taken. This value represents the average $F_{SHG}/B_{SHG}$ ratio of each individual cell counted in the entire cell ring ROI slice. The average $F_{SHG}/B_{SHG}$ ratio was then imported in R for the boxplots and the statistical analysis. To compare the $F_{SHG}/B_{SHG}$ ratios of the desired samples the Wilcoxon rank sum test was performed. The Wilcoxon rank sum test is a non-parametric alternative to the two-sample t-test that is solely based on the ranking the samples. This test is selected because the samples that are being compared are independent of each other and some of the samples are not normally distributed. In this test, the $F_{SHG}/B_{SHG}$ values are ranked to see whether they are evenly distributed or not across both populations, therefore, the original values are not directly considered. The p-value obtained assesses the probability of the null hypothesis which is the two populations are the same. For p-values $<0.05$ relates to the statistically significant difference in values across the two populations.

2.3 Result

2.3.1 Comparison of $F_{SHG}/B_{SHG}$ Ratio of Control Samples BJ and dBJ

The BJ fibroblast cells in the collagen gel model were stromal deactivated cells. And the dBJ fibroblast cells are the stromal activated fibroblasts with cAMP. Stromal activated fibroblasts that differentiate into myofibroblasts are useful for extracellular matrix remodeling for purposes such as wound healing, tissue inflammation etc. (as explained in Section 1.2.2). The (+) control collagen gel containing the decidualized (activated) fibroblasts (dBJ) shows an
increased $F_{SHG}/B_{SHG}$ ratio result. This increase indicates that there was a microstructural reorganization of collagen fibril packing. As discussed above in section 1.3.3, the increase in $F_{SHG}/B_{SHG}$ ratio comes from the phase matching effect and the fibrillar packing in the collagen environment. Based on visual observation of the resulting $F_{SHG}/B_{SHG}$ ratio image, it seems like there is higher collagen density in both (+ control) dBJ and (- control) BJ near the cell boundary in the ROIs, but remodeling as expected is seen highly in the activated fibroblast. However, there was not specific collagen density quantification done which can be part of the future work. The non-decidualized (deactivated) fibroblasts (- control) on the other hand did not induce remodeling in the collagen fiber compared to the decidualized fibroblasts (+ control). This result is also supported by the obtained lower $F_{SHG}/B_{SHG}$ ratio; dBJ sample has higher $F_{SHG}/B_{SHG}$ ratio than BJ sample. The $F_{SHG}/B_{SHG}$ ratios calculated assess collagen signatures locally in both the activated and deactivated fibroblast environments. The box plot (Figure 22) shows these differences in values for both control samples. The non-parametric test, Wilcoxon-rank sum test performed to compare the values of the $F_{SHG}/B_{SHG}$ ratios of both samples gives p-value <0.05 showing a statistically significant difference. From this we can understand that reactive stromal cell remodel collagen even without the presence of tumor cell.
Figure 22: The control gel model containing activated fibroblasts (dBJ) have higher collagen remodeling effect as seen in the heat map (A) than the control gel containing deactivated fibroblast (BJ), therefore has increased $F_{\text{SHG}}/B_{\text{SHG}}$ (B).
2.3.2 Comparison of F$_{SHG}$/B$_{SHG}$ Ratio of MCF7A and Inactive vs. Active Fibroblast

The experimental sample MCF7A_BJ contained the MCF7A cancer cell lines and the non-decidualized (stromal deactivated) fibroblasts which were left to interact with the cancer cells. This condition recapitulates the interaction between a cancer associated fibroblast and the cancer cell lines to induce remodeling on fibrillar collagen. The experimental sample MCF7A_dBJ contained the MCF7A cancer cell lines and the decidualized (stromal activated) fibroblasts. This condition recapitulates the interaction between the cancer cells and the stromal activated fibroblasts. The F$_{SHG}$/B$_{SHG}$ ratio obtained for the MCF7A_BJ sample has a larger median value than MCF7A_dBJ (Figure 23). This result shows that there is different microstructural reorganization of collagen fibril in tumor microenvironment where stromal inactive fibroblast (BJ) and MCF7A are interacting. This shows that the degree of remodeling in ECM protein by the interacting fibroblasts and MCF7A cancer cell lines varies based on the stromal cell condition (reactive vs. normal). Therefore, in MCF7A_BJ there is larger fibrillar packing highly ordered in the size scale of the SHG wavelength (as it resulted higher F$_{SHG}$/B$_{SHG}$ ratio) compared to MCF7A_dBJ. The crosstalk between the stromal active fibroblast and the ER+ cancer cell line does not seem as aggressive in imposing greater microstructural remodeling in collagen fiber compared to tumor/inactive fibroblast interaction. Qualitatively the collagen fiber appears to be highly straightened and aligned parallel to the tumor cell boundary in MCF7A_dBJ. However, the smaller F$_{SHG}$/B$_{SHG}$ ratio shows that the fibrillar packing in the microstructural orientation is not quite ordered to the size scale of SHG wavelength.
Figure 23: A. Heat map showing higher remodeling in collagen when MCF7A interacts with stromal activated fibroblasts than decidualized fibroblasts, therefore it results higher $F_{SHG}/B_{SHG}$ ratio (B).
2.3.3 Comparison of F\textsubscript{SHG}/B\textsubscript{SHG} Ratio of MDA\_MB\_231 and Inactive vs. Active Fibroblasts

The experimental sample MBA\_MB\_231\_BJ contains the cancer cell line MDA\_MB\_231 and the decidualized (stromal deactivated fibroblast) that are left to interact with the cancer cells. Again, in this environment the interaction between the aggressive cancer cell line and cancer associated fibroblasts is recapitulated. The experimental sample MDA\_MB\_231\_dBJ contains the aggressive cancer cell line and the stromal activated fibroblasts. This sample is where the remodeling in the fibrillar collagen by MDA\_MB\_231 cancer cells and the not specifically cancer associated but activated fibroblast is recapitulated. The remodeling induced on fibrillar collagen due to these interactions is quantified by the F/B ratio.

The F/B ratio obtained for MDA\_MB\_231\_BJ does not show a normal distribution in the values. This result is demonstrated in the heat map on the ROI image in which some areas are hotter than others (Figure 24). The hotter regions are associated with higher F/B ratio due to the higher fibrillar packing in the order of the SHG wavelength. The remodeling that activated fibroblasts induce on fibrillar collagen is highly characterized by pulling the fibers towards the cell surface.\textsuperscript{106} The model here sowing the remodeling induced by cancer associated fibroblast is a single mode of potentially multiple modes of remodeling i.e proteolytic degradation, tension mediated linearization, and collagen synthesis.\textsuperscript{107,108} In the image, it appears as if areas of higher collagen density surrounding the tumor border have a characteristics of shorter collagen fiber segments oriented perpendicularly to the tumor cell boundary. As part of future work, the fibrillar alignment can be quantified using a curvelet-based approach in addition to the localized F/B analysis of the microstructural fiber orientation.
The F/B ratio obtained for MDA_MB_231_dBJ does show a normal distribution in the values with a relatively higher median than MDA_MB_231_BJ. In this tumor microenvironment, the cancer cells, and the stromal activated fibroblasts (not necessarily cancer cell activated) both induce remodeling in the collagen surrounding them. Likewise, to the tumor microenvironment with cancer associated fibroblasts, there is higher collagen microstructural reorganization. Qualitatively it also appears that the collagen fibers are shorter in length but more spread out in their orientation near the tumor boundary. Based on a prior study, Bruke et al, demonstrated that higher F_{SHG}/B_{SHG} is associated with longer distance tumor migration, the localized F/B analysis for both MDA_MB_231_BJ and MDA_MB_231_dBJ presents tumor associated collagen signatures that seem to not favor tumor migration. These collagen signatures detected by the emission directionality ratio of SHG are associated with metastatic conditions. The remodeling behavior of the aggressive cancer cell MDA_MB_231 is different when cancer associated fibroblasts versus stromal activated fibroblasts are present in the environment.
Figure 24: MDA_MB_231 cancer cell line induces remodeling differently co-cultured with BJ versus dBJ (A). Although the F/B ratio of MDA_MB_231_BJ does not show a normal distribution statistically, it still has relatively lower median than MDA_MB_231_dBJ (B).
2.3.4 Comparison of $F_{SHG}/B_{SHG}$ Ratio of All dBJ Samples and BJ Samples

The localized F/B ratio analysis for the decidualized control and experimental groups has p-value $>0.05$. This result from the non-parametric Wilcoxon-rank sum test shows that there is no statistically significant difference between their F/B values. In these samples remodeling is induced both by the decidualized fibroblast and the two cancer types simultaneously. This result shows that a lower F/B ratio is associated with the presence of stromal activated (decidualized) fibroblasts in the tumor microenvironment. This low value is related to larger $\Delta K$ which describes the degree of phase mismatching and this shift in F/B could be due to on-going quasi-phase matching.$^{93}$ As described in Section 1.3.3, the F/B ratio is comprised from the subsequent forward and backward emissions from the dipole moment in the fibrillar assembly. The different collagen signatures from the remodeling are results of the induced reorganization of the fibers from their interaction with the cancer cells. The low F/B ratio quantifying this reorganization also gives information on the dipole moment organization within the collagen molecules in the fibrillar assembly.
Figure 25: TME containing decidualized (activated) fibroblast and both cancer cell lines (MCF7A & MDA_MB_231) induce disordered fibrillar packing in the collagen remodeling (lower F/B). Decidualized fibroblast & MCF7A in TME are characterized by more packed and aligned smaller diameter fibers. Decidualized fibroblast & MDA_MB_231 in TME are characterized by higher collagen deposition.
The localized F/B ratio analysis of the non-decidualized control and experimental samples have p-value <0.05. This result from the non-parametric Wilcoxon-rank sum test is showing a statistically significant difference between the control sample with a stromal deactivated fibroblast and the experimental sample with the two cancer cell lines. In the MCAF7A_BJ sample, where the stromal deactivated fibroblasts are actively interacting with the cancer cells, a higher F/B ratio value is obtained. In this scenario, the collagen signature is result of the reorganization by the cancer associated fibroblasts and the cancer cells. In this signature the dipole moment in the molecular assembly has lower degree of phase mismatching. The alignment and packing of the fibers here are ordered to the size scale of the SHG wavelength in the axial direction.
Figure 26: TME containing non-decidualized (deactivated) but cancer associated fibroblast & either MCF7A or MDA_MB_231 had a higher F_{SHG}/B_{SHG} ratio. In TME with MCF7A & cancer associated fibroblasts collagen is highly remodeled in which less randomly packed fibers are aligned. In TME with MDA_MB_231 & cancer associated fibroblasts collagen remodeling is characterized by fibers pulled towards the cell.
CHAPTER 3

DISCUSSION AND FUTURE WORK

3.1 Discussion

In a previous study completed by Bruke et al\textsuperscript{109}, different collagen microstructures were modeled to assess the behavior of tumor cells, however, the tumor cells themselves were not responsible for the remodeling. In their study it was shown that in tumor microenvironment where $F_{\text{SHG}}/B_{\text{SHG}}$ ratio is higher there is increased tumor motility. From this work, MCF7A_BJ sample having a greater F/B ratio than MCF7A_dBJ, shows that if the cells were being tracked there could be a possibility for the tumor microenvironment to be supportive of their motility. Furthermore, the non-parametric Wilcoxon-rank sum test performed to compare the $F_{\text{SHG}}/B_{\text{SHG}}$ ratio obtained for the MCF7A_BJ and MCF7A_dBJ shows a statistically significant difference with a p-value $<0.05$. In this condition it can be understood that MCF7A breast cancer subtype is most likely to metastasize in a non-reactive stromal cell environment where they are interacting with stromal deactivated fibroblasts.

Comparing the F/B ratio of MCF7A_BJ to MDA_MB_231_BJ, the reorganization of fibers locally near the boundary of the cells is hotter in majority of the areas as shown in the heat map in Figure 24. Whereas in MDA_MB_231_BJ, the F/B ratio heat map shows different ranges of values in $F_{\text{SHG}}/B_{\text{SHG}}$ ratio. This difference in F/B ratio depicts the fibril packing that SHG is sensitive to. The detected tumor associated collagen signatures in this localized F/B analysis is a very promising result to differentiate between all the presented conditions. Using this results the characteristic signatures resonating to the remodeling by the two different cancer cell types, by stromal activated fibroblast, and the stromal deactivated fibroblasts was understood. Furthermore, these results also show how the two difference cancer cells induce remodeling
when they are along with stromal deactivated (cancer associated fibroblasts) and stromal activated fibroblasts. The two cellular interaction types (reactive stroma vs non-reactive stroma with cancer cell) have different remodeling outcomes in their surrounding collagen fiber depending on the tumor microenvironment. The cancer cells themselves behave differently in remodeling fibrillar collagen around them when they are interacting with either of the fibroblast types. Relating F/B ratio from these signatures with previous studies completed on tumor motility based on F/B ratio variation, it can be claimed that the higher F/B ratio favors migration of tumor cells to secondary location.

SHG, as collagen specific imaging, is a promising tool to implement in a clinical diagnosis. This light-based technology gives deeper insight of the tumor microenvironment. Deeper understanding of the interaction between cancer cells and stromal cells is very important to identify the proper preventative action that needs to be taken to minimize the chance of the spreading of tumor. For that matter, several researchers have shown that SHG is a promising tool to be used for better patient prognosis in cancer studies.\textsuperscript{110-113} As briefly explained in Chapter 1, despite the presence of increased diagnosis in developed countries, the mortality rate of breast cancer has not decreased. The focus of these advanced diagnostic techniques is to reveal the unseen detail in tumor microenvironment. The results from this diagnosis are then used to determine the necessary treatment depending on the stage of the breast cancer. However, results from overdiagnosis can potentially lead to overtreatment. Therefore, the transition of advanced pathological instruments such as SHG from the bio-photonics society to the clinical team is impactful in minimizing overdiagnosis. For this bridge between bio-photonics lab research and clinical diagnosis to be successful the replication of laboratory testing protocols is crucial. The different biological models used in bio-photonics laboratories simulating a particular health
problem show the great potential in these technologies that is worth of translating to the clinical environment. Some of the current competitive instruments in the bio-photonics research world that had shown repetitive promising result in their tumor microenvironment study are listed in the following section.

3.2 Analysis of Imaging Techniques in Clinical Environments

In the field of medical imaging, the two main categories are optical imaging device and radiological imaging device. Optical imaging devices are used in a lot of primary diagnosis, point-of-care treatments, and in everyday medicine. Optical imaging is underestimated compared to radiological imaging, but it is a crucial component of healthcare. The advantage of optical imaging can be seen from two perspectives: clinical use and market analysis. A recent market analysis by Dr. Brian Pogue published on SPIE in 2018 has shown that compare to radiological imaging, optical imaging has a greater use in everyday medicine. The market analysis shown in the pie chart below (Figure 27) also shows that optical imaging has a bigger investment than radiological imaging in the global market. This pie chart shows that optical imaging plays the dominant role in the medical imaging market. However, as the market analysis of biophotonics presented by Stephen G. Anderson, SPIE’s industry and market strategist, the biggest challenge in photonics technology economic analysis is lack of data.
In the field of photonics, optical imaging tools are designed and used for a wide range of biomedical research to investigate several biological problems. These optical imaging devices most of the time are custom designed, and home built for specific in vivo or in vitro studies. Often these devices are built in an open space to modulate one standard design to several imaging techniques by simply switching some components. This method relatively is cost effective when compared to developing one optical imaging medical device. Most of the time these imaging modalities are built in research laboratories that are not necessarily clinical environment and so the open space development provides flexibility. If these devices are to be introduced to a clinical environment, they need to be built in a closed box. Furthermore, developing the device to be simple model but technologically complex enough to bring solution to a particular clinical problem impacts the adoption of the device to clinical use greatly. Another key consideration to take when developing the model is that it should be easy for the end user to
operate. Unnecessary complications on the model or requirement of special training to use the
device most of the time are great reasons for doctors, nurses, or other direct user healthcare
professionals to reject using the device. The merging of virtual reality with microscope has led to
the development of virtual microscope 20 years ago which contributed to the advancement of
digital pathology.\textsuperscript{116}

3.3 Current Slide Scanning Techniques For Clinical Use

Digital pathology has been playing a crucial role in the efficiency of clinical diagnosis
since its introduction 20 years ago.\textsuperscript{116} Since then, there has been several companies that have
been investing in producing slide scanning devices for clinical use. The current highly used
scanning devices in laboratories are whole slide imaging device. Philips Intellisite Pathology
Solution is the first whole slide imaging device to get the US Food and Drug Administration
510(K) clearance for clinical use without the need for premarket approval.\textsuperscript{117} Whole slide
imaging devices could either be manual or automated with the manual device being the cheaper
option. Although the manual whole slide imaging device are efficient and cost-effective, the time
consumption relative to the automated whole slide imaging devices is longer. The relatively
higher cost for automated whole slide imaging devices can be compromised by the greater
advantages it offers especially the speed in image capturing. Overall, the introduction of whole
slide imaging in digital pathology enhanced diagnostic ability, quality image production for
pathological assessment, advanced imaging, efficient time use, ability to process more slides, and
reduced physical data storage.\textsuperscript{118} Furthermore, operational cost analysis completed pre and post
implementation of whole slide imaging device has shown a significant saving in the overall cost
as shown in Figure 28 below. The current pricing for whole slide scanning imaging ranges from
$30k - $250k and the analysis shown in the graph below presents a saving of > $270k per year and $1.3 million in the overall five years analysis.\textsuperscript{118,119}

**Figure 28:** Operational cost analysis of whole slide imaging device considering every party that is involved from vendor to user (A) and overall cost saving analysis within a five year range (B). [Adopted from Lucas et al. (2021)]\textsuperscript{105}
The biggest determining factor when bringing a new medical device to the market is that it should not be more expensive compared to an already existing device. Therefore, optimization of cost of production is very critical for companies to consider. Not only cost of production is a determining factor but also cost for clinical trials and FDA approval is an additional constraint. Then, the challenge for the introduction of new optical imaging device to the market begins with clinical trial cost. Once all these steps are passed adoption of the device could be challenged by end users mainly pathologists (as a slide scanning microscope is considered as a pathological device), insurance companies (this takes the amount of money patients need to spend for the procedure into consideration), reimbursement strategies (if the device is to be adopted for clinical use this needs to be considered), and several market penetration constraints.

3.4 Advantages and Limitations of Digital Pathology

3.4.1 Advantage of Digital Pathology

- The Pathologists could easily communicate with other specialists from all over very quickly when reviewing patient’s slide.\(^{120}\)

- The steps in digital pathology are simple and easy: scan, digitize image, display. The scanning speed and the high-resolution digital images provides Pathologists with detail information within a reasonable time frame.\(^{121,122}\) The digitization of the images will minimize the need for physical storage of sample slides. Once the images taken from the slide scanning are digitized the images are saved and can be reviewed again without the need to view it under conventional microscope. This adds the value of being able to refer to patient slides at anytime it is needed. The displayed image has a spatial resolution in which the pathologist can view every detail in the slide.
Furthermore, this could also result in the development of computational models to assist the Pathologist in reviewing the slide for any cell analysis. Although, human trained personnel are always more efficient than computers, challenging tasks such as cell segmentation and particle analysis that are time consuming can transition from human to a trained computational model.\textsuperscript{103,123,124} This is not to say that machine learning can fully replace trained and experienced personnel, but the model can be objective and consistent to aid the work force.

- The current strongly growing digital pathology is whole slide imaging technology permits the collaboration of multiple specialized and sub-specialized people in reviewing a patient slide to decide on the needed treatment. This especially is very useful at times of public health emergency.\textsuperscript{119,125}

- The digitization of tissue section scans helps the pathologist to analyze any tissue section with a greater resolution to get detailed information.\textsuperscript{126}

- The advancement in this technology not only helps the clinical diagnosis but also medical research and preparation of standardized teaching materials for different disease states.

- In addition to the standardization of digitized tissue section analysis, whole scan imaging also benefits in quality assurance, retrieving data for electronic medical records, and improving the communication across institutions in patient treatment.\textsuperscript{127} This great clinical advantage comes from the fact the digital pathology cuts the need for physical transportation of tissue slides between expertise.

- Digital pathology is also useful for preparing educational materials using actual patient tissue samples instead of cartoons which aids the learning team greatly.
Furthermore, digital pathology brings students closer to clinical pathological samples for direct interactive learning without the need for physical slides.\textsuperscript{128}

- Digital pathology has a greater benefit for scientific research as it serves as a main source of digital images for the development of computational models for image analysis techniques applicable for clinical use.

- Applications of deep learning in academia has been greatly benefiting the research world but now the introduction of digital pathology helps in integrating computer aided diagnostics in pathological analysis in routine work. There are already companies that are working to develop AI technology for clinical use and have gotten FDA’s approval.\textsuperscript{129} The use of digital pathology helps in producing more digital images from the different disease states which serves as an input for the computational pathology to make generic models to analyze the images.\textsuperscript{130,131} The development of generic computational models to analyze digital images containing varied defective areas can enhance the efficiency of Pathologists. As a final step all the Pathologist has to do a confirm the detected areas by the computational model are the correct defective region.

3.4.2 Limitations and Challenges of Digital Pathology

- Initial high cost when first implementing digital pathology device is the number one challenge for laboratories.\textsuperscript{132}

- The price of digital pathology instruments is higher than having a simple light microscope that has traditionally been the gold standard to use in pathological labs.
The involvement of insurance companies and other governmental healthcare agencies to reimburse the clinic for these instruments is a critical need by any hospital.

- Adjustment of workflow to incorporate digital pathology to the already existing system could be challenging to the lab environment and to the team of experts. 133,134

- Although digital pathology has been discovered more than a decade ago, its adoption for clinical use is not growing at a faster rate. One of the many challenges in the adoption is the training of Pathologists to transition to the newer technology to maximize their productivity from the tedious traditional gold standard technique. The training by itself is challenged by the Pathologist’s self-assessment and the ability to learn faster. Just the replacement of microscope itself could challenge the Pathologists mentally since most experienced pathologists are old and are not prone to big changes. However, there are several laboratories sharing outcome and the process of transitioning to digital pathology. 118,132–136

- As digital pathology device are instruments that contain a relatively complex design than the simple traditional light microscope, any software and hardware issue could affect the workflow.

- Digital pathology requires higher capturing efficiency to digitalize, distribution, and storage of whole slide images. According to the current existing network and storage this might be challenging for every clinic to adopt digital pathology.

3.5 Market analysis for whole slide imaging

Since the introduction of virtual microscope, digital pathology has been capturing the attention of the pathology device market. Digital pathology has now more than five competitive
companies that are highly involved in the market. This highly growing industry is expected to reach $1,582 million USD in whole slide imaging device market.\textsuperscript{137} Analysis from a large health center after implementing digital pathology in full scale for five years has shown $18 million cost saving and same analysis in University Health Network in Canada shown $CA 131,000 cost saving per year.\textsuperscript{138,139} In addition to cost effectiveness, a critical component of the market analysis for whole slide imaging is the regulatory component. FDA considers whole slide imaging devices either as class III with a pre-market approval regulatory path or recently as class II \textit{de-novo} applications for limited use in immunohistochemical stained samples.\textsuperscript{140,141} Also, FDA released a guideline for testing of the whole slide imaging devices as they get introduced to for clinical use.\textsuperscript{142} Simplifying the steps in the FDA approval process for the whole slide scanning imaging devices is a determining factor for the development of market for digital pathology and also for the acceptance of the devices by pathologists. The development and approval of different image analysis techniques for pathologists to use, however, is increasing the adoption of digital pathology. Getting the most information out of a hematoxylin & eosin stained sample image is what pathologists care most about so developing this as cost effective and simple as possible would significantly increase the market.\textsuperscript{143} SHG as a label free slide scanning imaging technique adds a great value to the competitive digital pathology market if it can be commercialized in a cost effective way.

\textbf{3.6 Translational research for the commercialization of SHG}

In translation research where inventions go from bench to bedside, there are different kinds of considerations to take. Most of the time the work starts in academia research then it transfers to industry for development. However, often the culture and the work model of
academic research and industrial development go in their own separate ways. This gap between academic research and industrial development needs to be bridged with clinical consultation. Academic research completed without the cooperation with clinical needs and industrial business reality is challenged to successfully proceed in its translation to the real world. In the translation from academic research to industry then to clinic, there are three main factors: the type of topic, maturity of the idea (completion of basic research and proof of concept before transferring to commercial owner), and exist strategy (proper transferring to commercial owners and continuing connection). It is very important for the bio-photonics academic research team to consult with these two sectors if there ideas are medically applicable beyond academic laboratory.

3.6.1 Strength, Weakness, and Opportunity For Commercialization of SHG

3.6.1.1 Strength

SHG, as a slide scanning technique, belongs to the family of digital pathology technology. The current whole slide imaging techniques that are in clinical use do not have specificity in terms of sensitivity to any microstructures within the section of the imaged tissue. The whole slide imaging devices mainly simplify the tedious work in a pathologist lab but not necessarily advance the detection of changes in diseased and healthy tissue. The pathologist would still need to spend time looking into the images deeply for pathological assessment. Although there are some artificial intelligence programs that are being developed and approved by FDA for clinical use by companies such as Paige, the main focus is to help the pathologist identify suspicious areas faster and effectively. But imaging techniques that are sensitive to microstructural changes like SHG further simplify the pathological analysis process. A simple image analysis pipeline as shown in this work could be developed to differentiate between cases.
In modern life science, powerful imaging modalities used to study tumor microenvironment mostly require some form of labeling. SHG being a label free imaging modality is one of its strengths that makes it stand out from other techniques. The ability of SHG to give information on microstructural organization from the intrinsic property of a specific molecule is its great quality in tissue characterization and diagnostics. Recent study has shown that quantitative SHG analysis is regarded as a great addition to the qualitative morphological analysis of labeled tissue samples imaged using a bright field microscopy (a gold standard technique). The ability of this nonlinear optical imaging techniques to easily be incorporated into the traditional histopathological protocol advances the diagnostics process for cancer patients. Furthermore, studies completed by Shirmanova et al. and Wu et al. have showed that SHG can be used to characterize tumor associated collagen alterations after chemotherapeutic treatment. The outcomes from these studies show the strength in SHG imaging to serve as a diagnostic tool to differentiate between treated vs untreated tissue samples as well as different fibrotic responses in normal and treated carcinoma tissue. Moreover, a review completed by James et al. showed the promising future of SHG to be used as a diagnostic tool even beyond cancer.

3.6.1.2 Weakness

SHG is a powerful optical tool that if built based on clinical standards, it can significantly improve cancer diagnostics. One of the limitations in SHG that could possibly challenge the production of SHG for clinical use as a slide scanning device is that it has expensive cost of production. For SHG to be commercialized as a whole slide imaging technique, its production cost must fit in the estimated global whole slide imaging device market. The market of digital
pathology is at a growing rate, however, the cost of production for highly sensitive whole slide imaging instruments like SHG may be one reason to why it may slow down in its commercialization. However, this is not a discouragement for the possible future in SHG commercialization for clinical use.

3.6.1.3 Opportunity

Applications of SHG that are being shown in several biomedical research is an enlightening opportunity to commercialize SHG for clinical use. Companies like Harmonigenic™ Corporation are working on bringing SHG from lab to clinic to improve early treatment administration for people who are at early stages of cancer and those who have higher risk for metastatic cancer. The promising result they have accrued from using SHG to analyze primary tumor biopsies provided a prognostic information in ER+ and lymph node negative breast cancer patients. Seeing companies like this working towards introducing SHG for clinical use coupled with the growing market for whole slide imaging devices in the coming years shows possible opportunities for SHG commercialization. A study completed by Grubbs et al. showed the possibility for the production of a low-cost beam scanning SHG. Although in this research the produced SHG microscope was used in agrochemical materials imaging, this still shows the possibility for making a low cost SHG.
Bibliography


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APPENDIX

FIJI sample macro

// A for loop to calculate the F/B ratio.
for (i=0; i < Cell_Zproject.length; i++) {
    image=Cell_Zproject[i];

    // loads the segmented cell stack corresponding to FBSHG stack of each area.
    filename1= image + "BJC_Stack.tif";
    open(filename1);
    run("Enhance Contrast...", "saturated=10");
    setAutoThreshold("Li dark");
    run("Convert to Mask");
    rename("mask");
    run("Remove Outliers...", "radius=5 threshold=50 which=Bright");

    selectWindow("mask");
    run("ROI Manager...");
    run("Analyze Particles...", "clear summarize add");
    run("Divide...", "value=255");

    run("32-bit");
    setAutoThreshold("Li dark");
    run("NaN Background");
    rename("Cell");}
// Loads the folder containing the forward stack images.
filename2=F_stack + ".tif";
open(filename2);
rename("Forward");
setAutoThreshold("Li dark");
run("Fire");

// Loads the folder containing the backward stack images.
filename3=B_stack + ".tif";
open(filename3);
rename("Backward");
setAutoThreshold("Li dark");
run("Fire");

// Image math
imageCalculator("Divide create 32-bit stack", "Forward","Backward");
selectWindow("Result of Forward");
imageCalculator("Multiply create 32-bit stack", "Result of Forward","Cell");
roiManager("multi-measure append summary stack");

saveAs("Result", base_path + \results\FB_ + ".csv");
selectWindow("Results");
close();
run("ROI Manager...");
run("Close");
close("*");
AUTHOR’S BIOGRAPHY

Betelhem was born in Addis Ababa, Ethiopia in 1998. She was raised in the capital city of Ethiopia, Addis Ababa. She finished her primary, secondary, and high school in Nativity Girls’ School, a Catholic missionary’s school located in Piassa (a town in the capital city). She graduated from her high school on July 23, 2016 and came to the University of Maine for full study abroad January 10, 2017. She graduated with her Bachelor of Science in Biomedical Engineering in 2020. She entered the graduate school of the University of Maine to continue to her master’s in biomedical engineering in the Fall of 2020. After graduating, she will be working as a biomedical engineer either in a medical device production company or as clinical engineer. She is a candidate for Master of Science degree in Biomedical Engineering from the University of Maine in August 2022.