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# The use of second harmonic generation to image the extracellular matrix during tumor progression

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**Metastasis is the leading cause of cancer mortality, resulting from changes in the tumor microenvironment which increases tumor cell migration, dispersal to distant organs, and subsequent survival. This is accompanied by changes in tumor collagen which may allow cells to travel more efficiently away from a primary tumor and invade the surrounding tissue. Second Harmonic generation (SHG) is an intrinsic optical signal that has expanded our understanding of collagen evolution throughout tumor progression. This article addresses current research into tumor progression using SHG, as well as the future prospects of using SHG to advance our understanding of the tumor microenvironment.**

**Keywords:** collagen type I, multiphoton microscopy, Second Harmonic Generation, tumor progression, metastasis

**Abbreviations:** SHG, Second Harmonic Generation; PET, Positron Emission Tomography; MRI, Magnetic Resonance Imaging; MPM, Multiphoton Microscopy; ECM, Extracellular Matrix; TACS, Tumor Associated Collagen Signatures; TAFs, Tumor Associated Fibroblasts.

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## Introduction

Tumor metastasis is a multistep, low-efficiency process that remains the leading cause of cancer mortality throughout the United States.[1] Increasing our understanding of the dynamic changes that occur throughout tumor progression, and accompany the generation of metastases, may create opportunities to increase detection capabilities, personalize medical diagnostics, and develop new targeted therapies. As first suggested by Steven Paget with his "Seed and Soil Hypothesis,"[2] understanding tumor progression not only involves studying the tumor cells themselves, but also the cells and structures surrounding them. Consequently, studying the stromal changes throughout tumor progression is critical for understanding microenvironmental changes that may lead to increased metastasis. Current clinical methods of monitoring tumor progression are often incapable of monitoring stromal changes with adequate temporal and spatial resolution: Imaging of H&E or immunohistochemically stained tissue sections provides high resolution visualization of stromal changes occurring in the tumor, but only provides a "snapshot" of tumor progression in time. Other methods of intravital imaging, such as Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI), or ultrasound have improved tumor detection significantly over the course of the last few years, and can monitor temporal changes,[3-5] but lack spatial resolution and/or molecular sensitivity. The gap existing between these techniques is filled by intravital microscopy and especially multiphoton microscopy (MPM). In this perspective we will provide insight into the development and current role of Second Harmonic Generation imaging in the study of the stroma during tumor progression.

## Multiphoton and Second Harmonic Generation Imaging

In 1839 Rudolf Wagner pioneered the concept of *in vivo* imaging by using microscopy to study leukocyte interactions with vessel walls.[6] This revolutionized the study of biological processes, allowing for the analysis of tissue dynamics in real time. Fluorescence microscopy was invented in the early 1900 s, and its molecular specificity rendered it an important tool in the cancer biology field. However, this technique is not optimal for intravital imaging because of the high level of background fluorescence captured

when imaging a field within thick tissue.[7] This limits the signal to noise ratio as well as the imaging depth. The field of intravital microscopy took a significant step forward with the invention of the MPM, which enabled 3D imaging with high spatial resolution and significant depth penetration in tissue.[8] MPM has been used to excite both exogenous and endogenous fluorescence signals to study tumor progression *in vivo*, including studies of angiogenesis,[9,10] metastasis,[11–13] and extracellular matrix (ECM) modification.[14,15]

Within a few years of its development, MPM also became a tool for imaging intrinsic signals using the scattering phenomena of Second Harmonic Generation (SHG). In the process of SHG light is scattered by non-centrosymmetric structures to combine 2 incoming photons into one outgoing photon without the loss of energy due to a Stoke's shift as seen in fluorescence (**Fig. 1a**). Hence the outgoing photon has the same total energy as the incoming photons, and half the incoming wavelength. SHG was first discovered in crystals in 1961 [16] and later used to image collagen fibers *in vitro.*[17] In 1999 Campagnola *et al.* imaged cell membranes with SHG to monitor physiological changes in cancerous cells and healthy fibroblasts.[18] Soon after, SHG techniques were applied to human disease models of collagen rich tissue such as skin,[19] as well as in 3 dimensional collagenous tissue culture models.[20] Intravital SHG was subsequently exploited *in vivo* to image rat mammary adenocarcinoma progression through an acute skin flap[21] and to study collagen structure in human melanoma using a chronic mouse dorsal skin fold chamber, thereby initiating the use of SHG in the study of tumor progression.[22]

## Current Applications of SHG Imaging in Tumor Progression

SHG is currently applied *in vivo*, *in vitro* and *ex vivo,* most commonly to understand mechanisms of tumor progression as well as to attempt to detect and diagnose cancer based upon optical signatures (**Fig. 2**).

### Collagen reorganization in tumor progression

Tumor metastasis is commonly divided into several stages of progression including: development of the primary tumor, invasion of the tumor cells into the surrounding tissue, intravasation into blood or lymphatic vessels, survival of tumor cells in the vessel, extravasation from the vessel, and the development of a secondary tumor.[23-25] TPEF and SHG imaging of tumor progression often focus on the initial steps of this process, where SHG has the benefit of highlighting the changes in the stromal collagen structure throughout progression of the primary tumor toward metastasis. One of the pivotal findings in the field of SHG and tumor progression is that tumor cells travel along $SHG^+$ fibers as a means of collective or individual cell migration toward blood or lymph vessels.[21,26,27] Throughout breast tumor progression collagen fibers display a series of characteristic morphologies, entitled Tumor Associated Collagen Signatures (TACS),[26,28] which may affect the efficiency of metastasis. In TACS-1 collagen density is increased surrounding the tumor/host interface. These fibers straighten out to form a border circumferentially surrounding the tumor in TACS-2. In TACS-3 tumor collagen fibers are reorganized so that they protrude out perpendicularly from the tumor border, allowing cells to travel along the fibers toward surrounding blood vessels.[26] This SHG-based assessment of collagen morphology during breast cancer progression hints at a previously hidden dynamic control of collagen morphology and demonstrates the importance of studying the role of collagen in the tumor microenvironment to determine the mechanisms of tumor progression, with the goals of targeting this process as well as exploiting this in the clinic to assess tumor progression.

Collagen density, which correlates with tissue stiffness,[29,30] has been shown to affect the probability of developing breast cancer, as well as subsequently affecting the aggressiveness of that tumor.[31,32] Tumors exhibiting high stiffness show more metastatic tendencies, possibly as a function of their ability to reorganize the fibers to increase cell motility[26,33-35] and the abilities of individual cells to travel along these fibers.[36,37] SHG imaging is playing a major role in elucidating the mechanistic processes of ECM remodeling in the tumor microenvironment. One key regulator of tumor matrix stiffness is lysyl oxidase, which causes specific collagen crosslinking that has been shown, through SHG imaging, to result in the linearization of collagen fibers.[38] SHG imaging of tumor explants, xenografts, or individual cells seeded in collagen gels have shown that matrix reorganization is dependent on the ROCK[39,40] and FAK[41] pathways. SHG imaging of relatively "clean" collagen gel systems allows for parsing out these pathways in great detail, showing that significant molecules in these pathways include upstream Caveolin-1, which aids in remodeling the tumor ECM through the Rho/ROCK pathway.[36] In a mouse model of tumorigenesis in the involuting mammary gland microenvironment, inhibition of COX-2 reduces the collagen fibrillogenesis that is shown by SHG to be associated with involution, as well as the resultant tumor growth and metastasis.[42] On the level of organism-wide signaling, mouse models of emotional stress reveal that α2-adrenergic receptor activation promotes breast tumor progression and this progression is associated with alterations in collagen structure as shown by SHG.[43] Likewise, tumor collagen morphology can be altered by systemic application of the hormone relaxin, as demonstrated by *in vivo* SHG imaging.[22] *In vivo* SHG imaging also revealed that relaxin treatment increased the interaction of tumor associated fibroblasts (TAFs) with collagen fibers via TAF expression of β1 integrin, and this integrin expression is necessary for $TAF/SHG^+$ fiber association and subsequent fiber remodeling.[44]

Matrix metalloproteinases are enzymes responsible for matrix degradation and have been shown to play a significant role in tumor cell/ collagen interactions.[45,46] Mammary tumors developing in MMP13 knockout mice, a stromal MMP capable of cleaving collagen types I - III, have significantly elevated numbers of distant metastases as well as altered orientation

and structure, quantified through changes in the SHG signal directionality (see below). This revealed that MMP13 has a significant effect on tumor invasiveness and matrix structure.[47] SHG imaging of tumor cells in collagen gels has shown that membrane bound MMPs play a significant and necessary role in collagen reorganization and invasion. Membrane-anchored proteases, i.e. types 1, 2, and 3 metalloproteinases, aid in tumor cell penetration through the basement membrane as well as invasion through conventionally cross-linked tissue.[48,49] Although it is not yet clear what other stromal components are necessary for tumor invasion, it is very clear what a significant role SHG imaging is having on this field.

In addition to providing insight into the key molecular players, SHG imaging has also helped elucidate the cellular players expressing or responding to these enzymes and signals. Intravital SHG imaging has produced key insights into the related processes of mammary gland development[50] and mammary tumor progression,[51] and revealed a key role for the macrophage in both processes. The rate of tumor cell motility along $SHG^+$ fibers, and the rate of intravasation into blood vessels, was shown to be dependent upon proximity to macrophages.[52] Macrophages may also play a role in altering the structure of the fibers themselves: in a mouse mammary tumor model, ablation of stromal macrophages altered collagen structure as reported by SHG and reduced metastatic output, further implicating the macrophage and its collagen remodeling as a player in the metastatic process.[53] The aforementioned demonstration that the hormone relaxin promotes $TAF/SHG^+$ fiber association and fiber degradation suggests that macrophages may promote matrix remodeling via modulation of TAF behavior.[44]

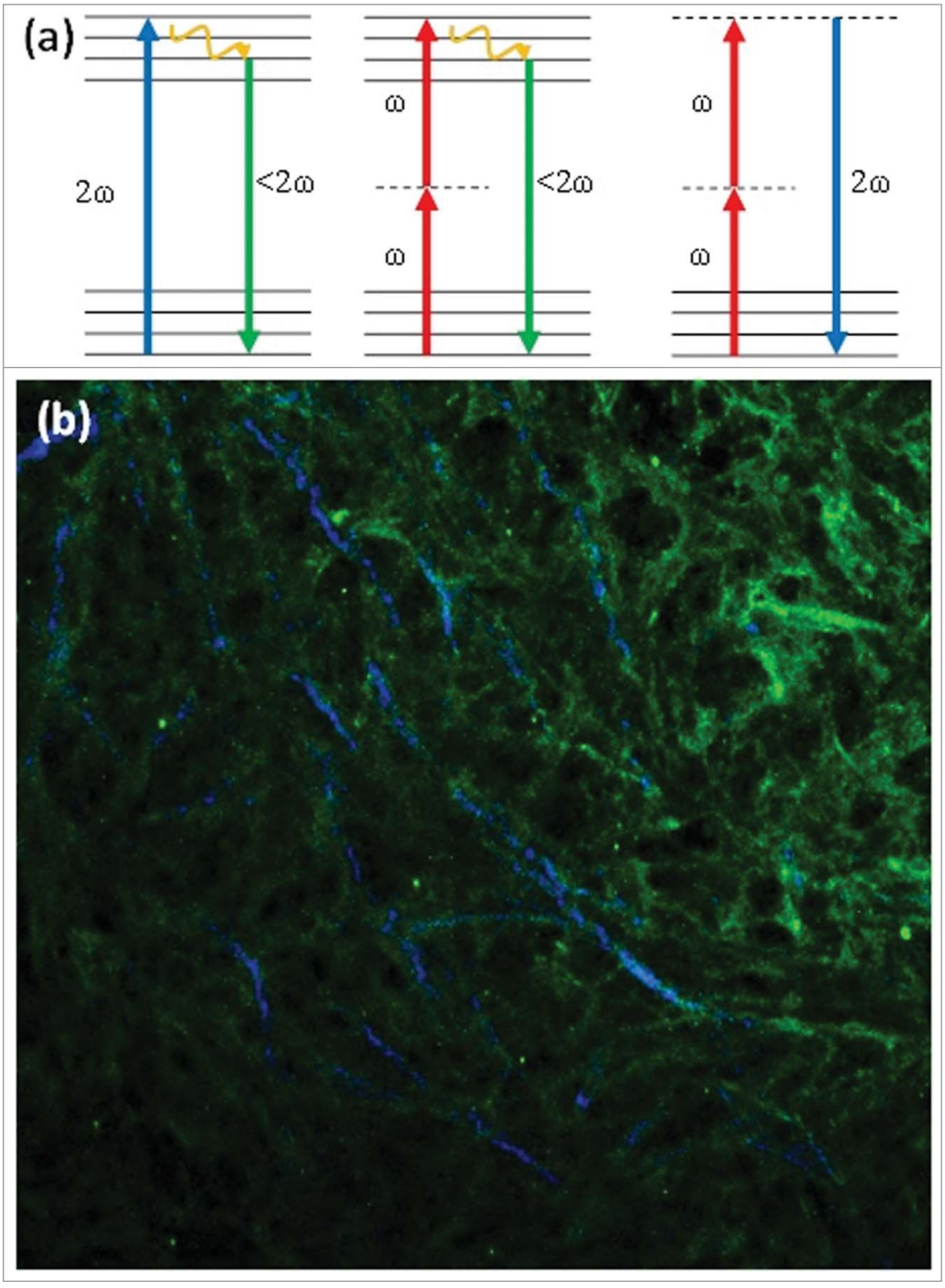


**Figure 1.** (**a**) Jablonski diagram of (from left to right) one-photon excited fluorescence, 2-photon excited fluorescence and Second Harmonic Generation, depicting the differences in excitation processes between these 3 optical processes. (**b**) Sample image of type I collagen antibody staining imaged with TPEF (green) overlapped with SHG imaging of collagen fibers (blue). This image demonstrates that SHG is produced by type I collagen, but not all type I collagen produces a significant SHG signal.

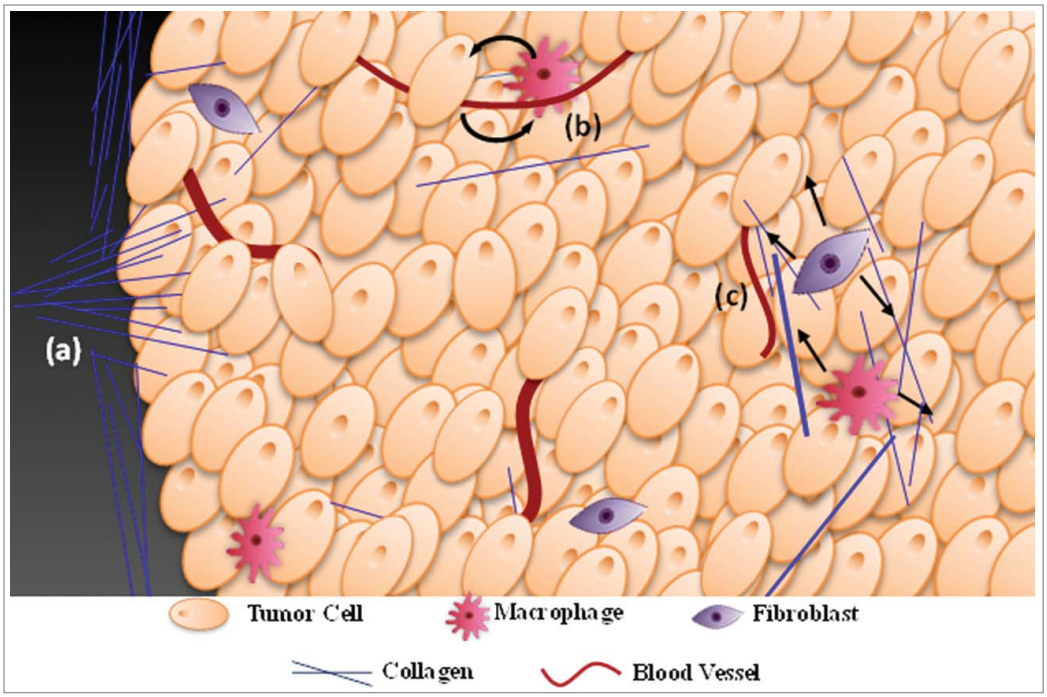


**Figure 2.** Summary of the major work being conducted in the application of SHG to tumor progression. (**a**) The tumor collagen framework undergoes significant restructuring which can increase the efficiency of cell travel away from the primary tumor. Morphological analysis of restructuring can subsequently be utilized to predict survival rates in breast cancer patients.[28] Furthermore, analysis of scattering directionality can be used to understand how matrix microstructure changes with progression. [63] (**b**) SHG is being used to monitor how tumor cells and host cells such as macrophages interact in the tumor microenvironment while using collagen as a framework to move toward blood vessels.[52] (**c**) Many *in vivo* and *ex vivo* studies are underway to better understand the pathways connecting tumor cells, macrophages, fibroblasts and the reorganization of collagen in the ECM.[44,53]

**Differentiating healthy and tumor tissue: morphology and SHG intensity**

SHG signals have been used not only to study the molecular and cellular regulation of collagen structure as described above, but also to differentiate between healthy and tumor tissue for a variety of different cancer types in an attempt to improve clinical detection and diagnosis of cancer. For example, SHG intensity and polarization properties were used to distinguish osteosarcoma, breast carcinoma, and melanoma from normal tissues.[54] SHG imaging of human tissue has been shown to be capable of differentiating between healthy and tumor tissue in breast cancer using collagen morphology.[55] Likewise basal cell carcinoma was distinguished from normal skin using the ratio of the intensity of the SHG signal to autofluorescence intensity.[56] Using the same ratio measurement healthy and tumor ovarian tissue have been differentiated *in vivo* through a laproscopic stick objective.[57] By analyzing the orientation of collagen fibers, as embodied in the Tumor Associated Collagen Signatures discussed above, one can predict survival rates of breast cancer patients.[28] The current field of SHG imaging has shown significant potential to increase clinical cancer diagnostic capabilities, by being able to locate areas of tumor presence within various tissue types, as well as to distinguish tumors by grade and metastatic ability.

*Differentiating healthy and tumor tissue: SHG directionality*

The methods described above face potential challenges in reproducibly exciting the same SHG intensity (for intensity based measurements) or in requiring trained observers or robust image analysis algorithms (to consistently outline fibers or analyze fiber orientation). An alternative method is to measure the directionality of the SHG signal. SHG emission is coherent, hence the directionality, intensity, and polarization of the outgoing light is sensitive to properties of the scatterers including scatterer order, spacing, angle, and the overall spatial extent of the scatterer distribution along the laser axis.[58–60] In collagen this translates to "microstructural" properties of the fibers, including: fibril diameter, spacing, and order versus disorder in fibril packing within the fiber.[58,59,61,62] These properties affect the directionality of SHG signals, which can be measured through the ratio of forward to backward propagating light (F/B ratio), which is inherently insensitive to variations in excitation efficiency. Measurement of F/B has been used to differentiate healthy and tumor tissue in ovarian cancer.[61] Recently, F/B ratio was used to differentiate invasive breast cancer from *in situ* breast cancer and healthy breast tissue, and was shown to vary with tumor stage and grade, revealing the power of this technique in a clinical setting.[63]

Measuring the true emitted F/B ratio *in vivo* is difficult due to the thickness of the tissue, because scattering of emitted SHG photons within the tissue affects the measured signals, and capturing a forward propagating signal intravitally can be essentially impossible due to the presence of tissue in between the plane of interest and the detection optics. Consequently direct measurement of the emitted F/B ratio is usually conducted using the direct capture of signals from thin tissue sections. Therefore it has been of interest in the field to explore methods to measure the F/B ratio from thick tissue sections, or even intact tissue. One method is to use collagen gels to study in detail how the measured F/B ratio changes as a function of depth, how different types of collagen types fibers affect the F/B measurements throughout gels, and hence how best to interpret forward and backward propagating detected SHG signals.[64] After measuring the F/B ratio as a function of depth

into a tissue with known scattering properties, Monte Carlo simulations can be used to extrapolate to the true F/B ratio.[59,61] As an alternative solution Han *et al.* developed a method to measure the F/B ratio using just the backward scattering signal by incorporating confocal pinholes of different diameters to determine the amount of forward propagating signal that is being scattered backward into the epi-detection lens.[65] This allows for the intravital measurement of the emitted F/B ratio, from the surface of intact tumor tissue, over time. The application of either thick tissue technique to intravital systems would aid in measuring microstructural changes in collagen fibers throughout tumor progression, *in vivo* or in explants.

## The Future of SHG in Tumor Progression

Based on the current progress in the field there are a variety of possible future applications of SHG in the study of tumor progression. Three of the most promising are using SHG in chronic window models to create and evaluate therapeutics that target the ECM, implementing SHG with molecular diagnostic techniques to produce more complete clinical diagnostics, and using SHG methods to find positive tumor borders on tumor biopsies.

### Creation and evaluation of drug treatments

The study of the cellular interactions and molecular pathways affecting matrix structure, as revealed by SHG and described above, has created a new window into the matrix and hence an opportunity to discover new therapeutic targets to inhibit metastasis. SHG has already been used to monitor tumor progression in animal models, consequently candidate therapeutic drugs can be administered in one of these models, and monitored with SHG, to determine how the candidate changes those aspects of collagen structure that influence SHG. This process is already starting, as demonstrated by the testing of Losartan treatment in mouse dorsal skin-fold chambers and the use of SHG to monitor changes in collagen structure, as well as subsequent therapeutic invasion into the tumor.[66] One possible class of therapeutic targets to explore with SHG is the membrane-bound MMPs. MMPs have been a potential drug target for over 30 y due to their obvious connections to matrix remodeling, but have thus far failed to produce efficient solutions for decreasing tumor invasiveness.[67] Through imaging of changes in type I collagen, membrane type 1 metalloprotease has been shown to play a necessary role in tumor invasion into the surrounding matrix ECM in covalently crosslinked collagen networks.[49] Membrane bound MMPs therefore provide a new target to explore for possible therapeutics using SHG, with the goal of inhibiting tumor invasion through the ECM through the use of these enzymes. Other alternatives to targeting MMPs include inhibiting the ROCK and FAK pathways, or their effectors, which have been shown to play significant roles in matrix reorganization throughout tumor progression.[39-41]

### Clinical evaluation through SHG and molecular diagnostics

With the increased capabilities of molecular diagnostics and genetic analysis, protein and gene expression profiles are beginning to play a more significant role in differentiating tumor characteristics and determining the best prospective treatments for a specific patient. As an example, OncotypeDX is a 21-gene screen used to help decide which patients will receive chemotherapy after removal of their primary breast cancer.[68] However, this is still an expensive and time consuming process. Many of the gene expression profiles known to indicate higher metastatic efficiency involve networks and pathways that control extracellular matrix structure.[69] Hence it is possible that SHG-based quantification of matrix structure may provide a readout that integrates the contribution of many of these networks and pathways. Consequently, correlating SHG properties with some of these genetic profiles could allow for a complementary, and possibly cheaper and quicker, method of providing predictions of tumor progression. For example, genetic testing has shown that the expression level of genes such as Snai1, and other genes related to epithelial to mesenchymal transition, are predictive of metastatic breast cancer, resulting from increased epithelial to mesenchymal transition of the cells.[70] Snail induces the expression of membrane anchored type 1 and type 2 MMPs on tumor cells, facilitating invasion through the surrounding collagen membrane,[71] a process that could be detected through SHG imaging. An SHG interrogation could integrate changes in gene expression without having to perform genetic testing, providing a quicker method of determining similar or complimentary information. Combining genetic research such as this with SHG imaging could increase diagnostic capabilities and aid in understanding the true physical consequences of the changes in genetic expression in the context of tumor development.

### Role of SHG imaging in tumor margin evaluation

As the clinical aim of breast cancer surgery transitions from mastectomy to lumpectomy for maximum preservation of healthy tissue, precisely defining the tumor margins and ensuring full removal of the primary tumor during the initial surgery have become increasingly important concerns in order to avoid secondary surgeries and cancer reoccurrence. The current standard for analyzing tumor margins involves removing and staining sections of the tumor, so a pathologist may analyze multiple sections to quantify negative, close, and positive tumor margins. This process, which requires several days, means patients will need to return for secondary surgeries if the borders are found to be positive for the presence of tumor tissue. Efforts to improve this process include various intraoperative techniques such as imprint cytology,[72] gross examination,[73] and ultrasound imaging.[74] However no extant method balances the accuracy, speed and ease of use required to achieve an ideal method of intraoperative analysis, and all present methods still result in 20–55% of removals requiring a secondary surgery.[75-77] The ability of SHG F/B to readily distinguish tumor from healthy tissue[61,63] coupled with the fact that it is a quantitative, intrinsic signal, suggests that it may provide useful

information to assist with margin assessment. Implementing F/B SHG techniques to detect different collagen profiles between healthy and tumor tissue along the border of the removed tissue would allow for the immediate analysis of the biopsy upon removal from the patient without the extensive staining and sectioning required for traditional pathological analysis. It has previously been demonstrated that *fresh*, human ovarian biopsies imaged with an MPM system showed differences in collagen morphology between healthy and abnormal tissue.[78] By combining this with quantitative methods of differentiating ovarian and breast tumor tissue from healthy tissue,[61,63] this could create an automated system of analyzing tumor biopsies for positive margins. The creation of a system that could rapidly image the surface of a tumor biopsy would hold the potential to significantly decrease the amount of secondary surgeries necessary after tumor removal, saving patients from unnecessary physical and emotional stress.

## Conclusion

Over the past 15 y Second Harmonic Generation imaging has advanced from its first imaging of cellular structures to routine use as an intravital imaging technique that can monitor cellular processes throughout tumor progression. Advancements in this field have illuminated necessary molecular pathways for collagen reorganization, increased knowledge about stromal evolution in cellular interactions, and created a new potential tool for cancer diagnosis. Many challenges still remain in regards to expanding our understanding of the tumor "soil," perfecting *in vivo* imaging methods, and translating this research to clinical applications. Overall SHG has proven to be a viable technique for identifying tumor location while providing a means of monitoring tumor progression intravitally with high spatial and temporal resolution.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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