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Two-Photon and Second Harmonic Microscopy in Clinical and Translational Cancer Research

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Abstract

Application of two-photon microscopy (TPM) to translational and clinical cancer research has burgeoned over the last several years, as several avenues of pre-clinical research have come to fruition. In this review, we focus on two forms of TPM—two-photon excitation fluorescence microscopy, and second harmonic generation microscopy—as they have been used for investigating cancer pathology in *ex vivo* and *in vivo* human tissue. We begin with discussion of two-photon theory and instrumentation particularly as applicable to cancer research, followed by an overview of some of the relevant cancer research literature in areas that include two-photon imaging of human tissue biopsies, human skin *in vivo*, and the rapidly developing technology of two-photon microendoscopy. We believe these and other evolving two-photon methodologies will continue to help translate cancer research from the bench to the bedside, and ultimately bring minimally invasive methods for cancer diagnosis and treatment to therapeutic reality.

Keywords

Two-photon microscopy; Cancer; Second harmonic generation; Collagen; SHG; Endoscopy

INTRODUCTION

Over the last 20 years, two-photon microscopy (TPM) has enjoyed explosive growth in its application to biomedical research. One of many areas in which two-photon (2P) microscopy has proven its utility is that of cancer research. In pre-clinical (i.e., laboratory models), translational, and clinical research, TPM has afforded a range of insights into cancer biology in areas related to tumor stroma and microenvironment; metastasis; angiogenesis; tumor metabolism, physiology and gene expression; and drug delivery and gene therapy. As is absolutely prerequisite to advance scientific knowledge to successful diagnosis and treatment of disease in humans, many of these studies have utilized TPM methods in pre-clinical cell and animal laboratory models, as that is where much of medicine begins. Much of this pre-clinical work has been elegantly reviewed in detail elsewhere, often focused on particular areas of cancer research, or on particular 2P methodologies (and of course, including relevant clinical studies as available and topically

appropriate). For example, Provenzano *et al.*⁹¹ capably discuss a range of research studies that have utilized multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to further our understanding of the tumor microenvironment and metastatic mechanisms. Several other excellent articles focus in whole or in part on reviewing intravital two-photon imaging investigations of tumor angiogenesis^{36,71,107} and metastasis.^{4,19,54,63} Other colleagues have discussed application of intravital TPM to studies of stromal cell dynamics and interactions in the tumor microenvironment,^{38,70} and to understanding the mechanisms and contributions of immune cells and the immune response to tumor pathology.¹²¹

To complement and extend these many capable works, we will begin with an overview of technologies, then will focus on implementation of two-photon optical microscopy primarily as applied to human biological materials—that is, either biopsied tissue (both fixed and fresh), or *in vivo* imaging—over a wide range of cancer types. A few pre-clinical studies will be mentioned when they relate closely to the clinical and translational studies surveyed. In closing, we will briefly highlight applications of TPM to human tissues in areas other than cancer. In this fashion, we hope to provide a broad overview of the demonstrated potential of two-photon optical microscopy instruments and methodologies as diagnostic and prognostic tools for human disease.

TWO-PHOTON MICROSCOPY THEORY AND INSTRUMENTATION FOR CANCER RESEARCH

As a principle of quantum mechanics, two-photon excitation was initially theorized by Maria Goppert-Mayer,³⁹ then first demonstrated in laser-excited crystals 30 years later,⁵³ and first applied to living biological specimens as TPM—thus triggering TPM's explosive growth in biological research—another 30 years after that.²⁵ As applied to biology, TPM utilizes customized, semi-customized, or turn-key commercial laser-scanning type microscopes, where the typical visible laser light sources [e.g., for confocal microscopy (CM)] have been replaced by pulsed, high-intensity near-infrared (IR) (usually ~680–1100 nm) lasers. Notably, the advent of commercially available turn-key tunable, mode-locked Titanium:Sapphire near-IR (~680–1080 nm) lasers with femto- to pico-second pulse widths greatly facilitated the widespread introduction of commercially available 2P microscopes, which in turn has enabled the expansion of multi-photon microscopy techniques to broader realms of biomedical research. Please see Zipfel *et al.*¹²⁵ and Rocheleau and Piston⁹⁸ for further technical background on TPM as a tool for biological research.

Two-Photon Excitation Fluorescence (TPEF) vs. Second Harmonic Generation (SHG) Microscopy

In the most common TPM technique, sometimes referred to as TPEF microscopy, an exogenous or endogenous fluorescent molecule is excited by the near-IR laser, and the resultant fluorescent signal is recorded by photomultiplier tubes (PMTs), thus providing a means of optically monitoring physiologic and biochemical events within the cells or other biologic tissue. In addition to the two-photon excitation of a fluorophore and subsequent monitoring of the fluorescent emission signal (i.e., TPEF), TPM can be also be utilized to take advantage of other physical and optical properties of two-photon interactions with tissue. For example, another TPM technique increasingly applied to cancer research is SHG microscopy. SHG, like TPEF, also depends on two photons interacting simultaneously with a target. Unlike TPEF, however, in which two photons are absorbed by the target to produce a single photon of fluorescent emission (with some energy loss), with SHG there is no *absorption* of photons by the target. Rather SHG involves *scattering* of photons and no energy loss, whereby two photons interacting simultaneously with a non-centrosymmetric

target combine to produce a new photon with exactly twice the energy (thus twice the frequency, and half the wavelength) of the interacting photons^{10,81,83,95} (Fig. 1). Hence, 2P irradiation of an SHG generating target at 810 nm will emit SHG photons at 405 nm.

These differences between TPEF and SHG notwithstanding, here it is also important to note that because of the *similarities* between TPEF and SHG, both of which involve laser-induced two-photon interactions with the imaged subject matter, the majority of TPM hardware and technical considerations discussed below apply equally to TPEF and SHG microscopy, with the fundamental difference being that spectrally distinct emission filters are used to capture the disparate SHG and TPEF signals. Therefore, for clarity herein, we will use “TPEF” to refer specifically to TPEF microscopy, “SHG” to refer specifically to SHG microscopy, and “TPM” to refer generically to TPM features which are mutually applicable to both distinct methodologies. In addition, while TPEF microscopy can utilize either exogenous or endogenous (intrinsic) fluorescent molecules, herein we will primarily discuss TPEF signals arising from the numerous intrinsic fluorophores found in biologic tissue.

Instrument and Image Acquisition Considerations for Clinical and Translational Cancer Research

As laser scanning microscopy techniques, the laser-substrate interactions and emission capture (of TPEF and/or SHG signals) in TPM typically occur one pixel at time, by raster-scanning the laser focal point over the designated XY specimen area within the microscopic field of view. As such, typically configured TPM generally achieves <1 frame/second. However there are numerous reports of video rate (30 frames/second) and super-video rate (usually by multi-focal approaches,^{1,5,6,34,55,72,104}) TPM enabled by hardware customization to improve scanning speeds, and many of these methods could conceivably be applied to clinical and translational cancer research. Video-rate or better TPM may ultimately prove useful for imaging human cancer tissue physiology in real-time, and/or for high throughput imaging of archival tissue specimens.

The pulsed, near-IR laser is in fact integral to application of TPM in living biological tissues. In conventional one-photon fluorescence excitation, a single-photon excitation of a fluorophore results in the emission of a single lower energy photon of longer wavelength (i.e., the Stokes shift). Two-photon excitation theory holds that when irradiance is of sufficient intensity (MW-GW/cm²),⁶⁰ two photons interacting simultaneously or nearly simultaneously ($\sim 10^{-16}$ s)²⁵ with a fluorophore will produce a single photon of fluorescence, equivalent to if that fluorophore had been excited by *one* photon of twice the energy (i.e., half the wavelength) of each of the two exciting photons. Thus to achieve 2P fluorescence excitation, longer near-IR (i.e., double wavelength) lasers substitute for the shorter visible spectrum lasers or light sources typically used for one-photon excitation fluorescence microscopy (e.g., confocal and widefield fluorescence microscopy). To obtain the high light intensities required to achieve 2P fluorescence excitation, near-IR lasers typically produce very brief pulses (usually ~ 150 femtoseconds or less) at high Hz, which creates sufficient *peak* (instantaneous) energy to produce 2P excitation, while keeping *average* energy low enough to minimize specimen damage.¹¹⁰ In this fashion, these rapid, ultra-brief high power pulses, together with focusing of the laser through the objective lens, ensure that photons are sufficiently “crowded” in space and time to enable 2P interactions to occur.⁹⁸ This means that in TPM, laser excitation of fluorescence is confined exclusively to the microscope objective’s focal volume, because only here is there sufficient photon density to cause significant 2P excited fluorescence. Thus unlike other fluorescence approaches (e.g., confocal, widefield), 2P excitation light does not cause significant “stray” fluorescence as it passes through tissue above or below the focal plane, thus greatly reducing photobleaching and photodamage in the specimen and eliminating the need for a confocal pinhole, enabling

imaging deeper into tissue.^{98,125} Moreover in tissue, near-IR scatters less than shorter wavelengths (facilitating deeper penetration), and are less damaging than shorter wavelengths. All these features make TPM ideally suited for live biologic imaging. Still important to note however, is that while these aspects of TPM greatly reduce the potential for “out of plane” photodamage and phototoxicity compared to one-photon microscopy approaches (e.g., CM), they cannot eliminate “in plane” photodamage, because in order to generate TPEF signal photons “in plane,” one must by necessity generate photobleaching. This “in plane” photobleaching is mitigated by the lack of confocal pinhole and the accompanying increased sensitivity, resulting in fewer signal photons (and photobleaching events) generated per detected photon, relative to confocal imaging. Thus particularly when considering any TPM approach for *in vivo* and clinical imaging, due attention must be given to both the peak and total energy exposures of the specimens, so as to minimize potential tissue damage and phototoxicity related imaging artifacts.

Many excellent references discuss TPM theory and instrumentation in further detail.^{5,6,25,60,87,98,110,125} Herein we will focus principally on the application of TPM to clinical and translational cancer research, referring to our colleagues’ works for additional background.

MONITORING INTRINSIC BIOLOGIC SIGNALS BY TPM

The fluorophores excited and monitored in TPME can be either exogenously applied—e.g., fluorescent dyes, fluorescent reporter proteins such as green fluorescent protein (GFP), or fluorescently labeled antibodies—or they can be fluorescent molecules endogenous to the tissue. In pre-clinical *in vitro* and *in vivo* research models, such exogenous fluorophores are commonly used to investigate molecular events by TPM. In clinical and translational cancer research, although exogenous fluorescent molecules can be employed—for example, staining of archival tissue by fluorescently labeled antibodies, or administration of tracer dye molecules for *in vivo* imaging—frequently investigators are seeking to track endogenous fluorescent signals by TPM to gain information about extra/ intracellular processes and interactions in normal vs. disease states. Therein lies one of the strengths of TPM: the ability to investigate intrinsic biologic activity *in vivo* in real time, with near-IR excitation, without the need for additional extrinsic marker molecules that may be toxic or otherwise interfere with normal metabolic activity. For investigations of cancer biology, particularly as applies to *ex vivo* and *in vivo* imaging of human tissue as is our focus herein, this ability to monitor intrinsic metabolism becomes particularly appealing, with the idea that these signals may ultimately yield diagnostic or prognostic clues about cancer. Therefore, and because usage of exogenous fluorophores in pre-clinical cancer research models has been well covered elsewhere (see references in “Introduction” section above), herein we will focus our discussion principally on endogenous fluorescent markers and other optical phenomena that can be monitored by TPM in the context of clinical and translational cancer research.

Endogenous Fluorophores

Biological tissues contain many molecules endogenous to normal cellular biochemistry that also happen to be fluorescent, and this fluorescence can therefore be exploited by researchers to provide a window into cellular activity. Some of the endogenous fluorescent molecules found in tissues, and that have been fluorescently imaged, include: the mitochondrial matrix proteins nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), and lipoamide dehydrogenase; the structural proteins elastin, keratin, and collagen; and the pigments lipofuscin, melanin, and porphyrins (these and other intrinsic fluorophores are thoroughly reviewed in Wang *et al.*¹¹⁰ and Zipfel *et al.*¹²⁴). The fluorescence of these molecules often arises from smaller fluorophores that comprise their structure, such as the UV-emitting amino acids tryptophan, tyrosine, and phenylalanine, and

the 350–600 nm emitting vitamins or vitamin derivatives such as retinol (form of vitamin A), riboflavin (vitamin B2), niacin (vitamin B3) and pyridoxine (vitamin B6), folic acid (vitamin B9), and cholecalciferol (vitamin D3).¹²⁴ A majority of intracellular fluorescence arises from NADH, flavins, retinol, tryptophan, and indoleamine derivatives of tryptophan such as serotonin and melatonin, whereas most extracellular fluorescence arises from the structural proteins elastin and collagen.¹²⁴ TPEM of NADH and FAD, and both TPEM and SHG microscopy (see next section) of collagen, have arguably been the most investigated intrinsic fluorophores in cancer research. (NADH and FAD have also been heavily investigated in cancer by 1P microscopy approaches, although below we focus our discussions primarily on 2P investigations of these intrinsic fluorophores in cancer research.) As fluorophores, any of these molecules can be excited by either 1P or 2P excitation. However, the benefits of 2P over 1P microscopy for tissue imaging described above, and because the 1P excitation spectra for most of these compounds falls in the tissue-damaging ultraviolet (UV) range, makes 2P excitation ideal for imaging these molecules in living tissue.

In addition to capturing the intensity or emission spectra of these intrinsic biologic fluorophores, the *fluorescence lifetime*, which is sensitive to binding states, molecular interferences, and other aspects of the molecular environment,^{3,16,67,91,109,120} can be measured using FLIM. Thus, TPM FLIM data can provide important information about the local biochemical milieu that may aid in distinguishing benign from pathologic cancer features, and as such FLIM is being increasingly employed as a tool in TPM cancer research. A number of elegant discussions and detailed demonstrations of FLIM's utility in cancer research have been presented,^{21,91,94} including for human skin imaging,⁹⁷ and thus we won't cover this topic in great detail here. However, below we do discuss some FLIM findings as they otherwise fall under our topical criteria, i.e., studies of cancer pathology in *ex vivo* and *in vivo* human tissue.

Second Harmonic Generation

As described above and in Fig. 1, SHG is another two-photon interaction whereby rather than producing a fluorescence excitation, two photons instead interact simultaneously with a non-centrosymmetric target to produce a new photon with exactly twice the energy (i.e., twice the frequency, and half the wavelength) of the interacting photons.^{10,81,83,95} Hence this emitted SHG signal can be generated and monitored using TPM equipment, and is frequently collected in parallel with a TPEF signal, thus providing another means by which TPM can capture biochemical information from intrinsic biologic molecules.

Both TPEF and SHG are often referred to as “nonlinear” optical processes, because the intensity of their emission signals (i.e., the rate at which 2P interactions occur) is proportional to the square of the irradiating intensity. In other words, doubling the irradiating intensity produces four times the emission signal (TPEF or SHG). Contrast this with 1P excitation, in which the fluorescence emission scales linearly with the excitation intensity. Because SHG and TPEF are nonlinear processes, they are *similar* in that they are spatially confined and hence highly useful for 3D imaging and can be performed simultaneously with TPM. They are *different* because SHG is coherent, and hence the amount, direction, and polarization of SHG emission is strongly influenced not just by the scatterers' concentration, but also by their spacing, order vs. disorder, and orientation (Fig. 2). Thus, SHG provides a window into molecular structure not readily available with TPEF alone. Please see Campagnola and Loew,¹⁰ Nadiarnykh *et al.*,⁸² Han and Brown,⁴³ Han *et al.*,⁴⁴ and Campagnola⁹ for detailed overviews of these concepts.

In the context of cancer and this review, collagen, of which there are many forms, is a highly ordered non-centrosymmetric extracellular matrix (ECM) protein that generates a strong

SHG signal (Fig. 3), and a significantly weaker TPEF signal. In purified gel form, the strength of collagen's SHG and TPEF signals vary with the irradiating wavelength, with 800 nm irradiation producing the strongest SHG (400 nm) and a weak TPEF (~450–600 nm emission, with peak at ~525 nm), whereas 730 nm irradiation produces the strongest collagen TPEF (same emission spectra) and a relatively weaker (but still potent) SHG signal (365 nm) compared to 800 nm irradiation.¹²⁶ Collagen was first identified as an SHG-generating component of biological tissue in studies from two groups 20–30 years ago.^{30,100} However only with the serious emergence of TPM in biological research did capturing of the SHG signal from collagen and biological tissues become more readily accessible.^{8,11,12,22,41,56,106,124,126} Several early pre-clinical studies utilized TPM to image SHG and collagen in *ex vivo* or *in vivo* tumor tissue,^{8,42,112} and since then TPM SHG has become a widely employed tool for probing cancer biology, since probing collagen's molecular organization may provide important insights into cancer pathogenesis.

Precisely how and what features of collagen molecular organization affect SHG are topics of significant ongoing investigation, and beyond the scope of this review. Suffice to say for the moment that SHG is enhanced by non-random (and not anti-parallel^{33,103}) molecular organization (which promotes constructive enhancement of the vectorial SHG signal, rather than destructive interference), and by ordering on the scale of the irradiating wavelength.^{10,81,82,103} Polarization of the SHG signal, and the forward to backward (F/B) ratio of the emitted SHG, can also provide information about the orientation of individual SHG scatterers, and the length scale over which ordering occurs, respectively.^{43,44,82,116} Readers can refer to those cited references for technical detail, but for the purposes of this discussion, the key points are that collagen's concentration, spacing, order vs. disorder, and orientation will influence the amount, direction, and polarization of its SHG emission (Fig. 2).

UTILIZATION OF TPM IN CLINICAL AND TRANSLATIONAL CANCER RESEARCH

In this section, we seek to provide a useful summary of some of the findings enabled by TPM on human clinical tissue in cancer research. With quite a few papers meeting these criteria published in the last few years, we will particularly strive to cover some of this more recent work. Our discussion will be organized going “up the ladder” of clinical and translational research with human specimens, from *ex vivo* biopsied tissue (organized by imaging target, e.g., SHG, NADH, *etc.*), to real-time imaging in human subjects. Included in this latter section will be discussion of some evolving TPM technologies for human imaging—e.g., subsurface TPM endoscopy. Finally we will briefly discuss future directions for TPM imaging of human disease.

TPM in Human Biopsied Tissue

Most often, the endpoint goal of biomedical research is to translate and evolve findings from pre-clinical laboratory research to treatments for human disease. As part of this process, many pre-clinical research investigations ultimately lead to studies of *ex vivo* or *in vivo* human tissue, and ideally to clinical drug or therapy trials as findings warrant. That we are able to discuss applications of TPM in the clinical and translational research findings relayed here is a testament to the wealth of excellent pre-clinical research that has made these studies possible.

Quantitative SHG of Collagen

Many of the pre-clinical cancer studies utilizing TPM have been aimed at understanding the role of collagen and other intrinsic molecular markers in breast cancer (reviewed in

Provenzano *et al.*⁹¹ and Provenzano *et al.*⁹²). It is theorized that organization or reorganization of collagen, a key component of the ECM, may play roles in tumor genesis, progression, and/or metastasis.^{8,44,47,90,101,118} As discussed above, differences in collagen organization, in turn, may be realized as measurable differences in SHG characteristics (Fig. 4). Significant pre-clinical research helped lead to a recent extensive analysis of collagen SHG in biopsied human breast carcinoma tissue. In an analysis of microarrayed tumor tissue cores from 196 breast cancer patients, Conklin *et al.*²⁰ found that an increased presence of collagen fibers aligned perpendicularly to the tumor boundary (as determined by SHG signal) was associated with decreased survival. Moreover, inclusion of SHG intensity data (to provide some measure of the amount and/or ordering of collagen fibers), rather than just visual assessment of the “directionality” of the SHG signal relative to the tumor interface, slightly increased the significance of this effect.²⁰ In pre-clinical work in a mouse model of breast cancer, this same group had previously termed this kind of collagen arrangement (i.e., oriented perpendicularly to the tumor boundary) a “tumor-associated collagen signature-3” (TACS-3), whereas TACS-2 indicated straight “taut” fibers often parallel to the tumor boundary, and TACS-1 reflected the presence of dense collagen near small tumors.⁹⁰ Because tumor cells preferentially migrate along aligned collagen fibers,^{93,113} it is hypothesized that progression in collagen organization sequentially through the TACS-1–3 stages may underlie the transition to tumor metastasis.^{20,90} Together these findings help solidify a clinically relevant role for collagen and SHG as biomarkers with prognostic value for predicting breast cancer outcome.

Other work has provided detailed quantitative analyses of differences in SHG properties between normal and cancerous human ovarian biopsy tissue. In this report, Nadiarnykh *et al.*⁸² found that compared to normal tissue, malignant ovarian tissue was characterized by denser and more ordered collagen, as determined by qualitative analyses of collagen SHG patterns, and by quantitative analyses of SHG intensity (higher in cancer), and of the scattering properties of the SHG signal (higher in cancer). The SHG F/B ratio was found to be lower in the malignant tissues, again consistent with denser and more regularly packed fibrils that would more efficiently backscatter the SHG.⁸² This work provides useful quantitative methods and guidelines for using SHG to distinguish healthy from cancerous tissue. Another study displayed similar differences in collagen morphology and structure in ovarian vs. normal tissue, which they quantified as an increased SHG pixel uniformity in the cancer tissue, reflecting a transition from discrete, randomly oriented linear fibrils to a wavy, more uniformly organized collagen network in the normal vs. cancer tissue, respectively.⁵⁸

Studies have used SHG to differentiate cancerous from normal tissue for other cancer types as well. To control or normalize for artifacts unrelated to experimental effects, many of these studies have expressed the SHG signal as a ratio, relative to some defined or undefined tissue autofluorescence signal acquired at the same excitation wavelength. For example, in a pilot study, by defining their multiphoton autofluorescence (MAF) to SHG index (MAFSI) as MAF-SHG/MAF+SHG, Wang *et al.*¹¹¹ found lower SHG (i.e., MAFSI closer to “1”) in lung adenocarcinoma (LAC) and squamous cell carcinoma (SCC) compared to pair-matched normal lung tissue from the same patient. Likewise, by performing multiple analyses of SHG and TPEF pixels in their TPM images, Zhuo *et al.*¹²² found several significant differences in normal vs. neoplastic human esophageal stroma. In brief, compared to normal esophageal stroma, neoplastic stroma displayed: 1. A less defined, more diffuse collagen fibril structure (as determined by how rapidly neighboring SHG pixels “fall off” in value); 2. Loss of collagen (i.e., reduced SHG pixel area); 3. Reduced spacing between elastin fibers; 4. Increased elastin area, and 5. Reduced ratio of collagen to elastin (i.e., SHG/TPEF) signals.¹²² In these studies, the TPEF signal at 850 nm excitation was attributed to elastin (TPEF from collagen is negligible at 850 nm¹²⁶), and could be seen from 450 to 625+ nm with a peak at ~510 nm.¹²² A similar recent study by many of the same authors also found

reduced collagen area (i.e., ratio of SHG pixels to total pixels) in cancerous compared to normal gastric tissues.¹⁷ Fortunately, although collagen and elastin have some overlap of their TPEF excitation and emission spectra, they can be most readily distinguished because only collagen will produce SHG.^{15,17,122,124}

Optical Biopsies

Other studies have been more qualitatively focused on achieving “optical biopsies,” endeavoring to characterize and map intrinsic fluorescent optical signals for correlation of tissue architecture and pathology with “gold standard” hematoxylin-eosin (H&E) stained histopathology slides. If successful, TPM may eventually provide a more rapid, real time substitute for traditional histopathological processing and analyses. In *ex vivo* intact human gastrointestinal mucosa, using TPM of intrinsic fluorescent signals alone, Rogart *et al.*⁹⁹ found they could discern a level of structural detail similar to that found in H&E slides, and that TPM was superior to CM for these purposes. Similar conclusions were drawn in comparative analysis of TPM-imaged and H&E stained normal and cancerous human ovarian biopsy tissue, and this study went on to also identify several parameters uniquely discernible by TPM (e.g., collagen SHG changes, and red-shifted cellular intrinsic fluorescence) by which they could distinguish normal from cancerous tissue *in vivo* in a pre-clinical mouse model of ovarian cancer.¹¹⁵

In another study of gastric tissues, Yan *et al.*¹¹⁹ found they could distinguish cancer from control gastric tissue with simple qualitative comparisons of observed TPM SHG and TPEF features, which also correlated well with common identifying characteristics on the equivalent stained histopathology slides. Similar observations were made using TPM of intrinsic signals alone to distinguish between normal, nodular goiter, and papillary cancerous thyroid tissues.⁵⁰ In a similar but somewhat more detailed study, Tewari *et al.*¹⁰⁵ used intrinsic TPM optical signals gathered at 780 nm excitation and 3 emission bands—355–425 nm (SHG), 420–530 nm (short wave autofluorescence that captured mostly elastin, NADH, and FAD in these tissues), and 530–650 (long wave autofluorescence, theorized to capture lipofuscin signal)—to positively identify all key prostatic and periprostatic anatomical structures, and distinguish between normal, benign hyperplastic, and cancerous prostate glands. Finally, in non-human tissue, a similar detailed approach identified normal and cancer pathology in mouse lung tissue using intrinsic TPM signals.⁸⁶ Pre-clinical animal optical biopsy work has also identified oral cancer as an area which may ultimately benefit from *in vivo* TPM diagnostics in the clinic.^{37,114} With the continued development of TPM endoscopy, these findings are particularly significant, as they harbingers the possibility of real-time *in vivo* TPM diagnostics of cancer.

NADH and Metabolic Analyses

Several intrinsic intracellular fluorophores can provide information about cells’ metabolism and oxidative reduction capacity. Since tumor growth is an energyheavy process, this metabolic information may in turn prove useful for distinguishing cancerous vs. normal tissue. NADH and FADH₂ (reduced FAD, i.e., flavin adenine dinucleotide hydroquinone) are two reducing agents (electron donors) instrumental in mitochondrial production of ATP by oxidative phosphorylation within the electron transport chain. Because NADH and FADH₂ are both produced and utilized (i.e., oxidized) during the process of cellular respiration, relative concentrations of the oxidized and reduced forms can provide an overall measure of cellular metabolism. NADH is only fluorescent in its reduced form (NADH), whereas FADH₂ is fluorescent mainly in its oxidized FAD⁺ form, and these fluorescent forms can be imaged by TPM.^{89,124} Thus utilizing these captured signals ratiometrically, often referred to as the “Redox ratio” (i.e., reduction oxidation ratio), can provide a measure of cellular metabolism. This Redox ratio has been expressed in many forms, but when

expressed as $[FAD+]/[NADH]$, a decrease in this ratio typically corresponds to an increase in overall cellular metabolism.^{13,102} Because these techniques measure metabolic status, live *ex vivo* tissue is usually used for real-time physiological assessment, although useful NADH and FAD TPM measurements can apparently also be obtained from fixed tissue.²¹

In fresh human bladder biopsies imaged within 1 h of extraction, Cicchi *et al.*¹⁸ found a decrease in both the Redox ratio (expressed as $FAD-NADH/FAD+NADH$) and the fluorescence lifetimes of NADH and FAD, in cancerous compared to healthy bladder mucosa. The authors were also able to differentiate cancerous vs. normal tissue by an index of SHG (445 nm) and TPEF (475–620 nm) signals, expressed as $SHG-TPEF/SHG+TPEF$, with this index being lower in carcinoma *in situ* bladder tissue. In contrast, in fresh ovarian tissue biopsies, Kirkpatrick *et al.*⁵⁸ found their redox ratio $FAD/FAD+NAD(P)H$ was lower in normal “low risk” tissue, trended higher in normal “high risk” tissue, and was significantly *higher* in cancerous ovarian biopsies. Since *lower* redox ratio has commonly been associated with cancerous tissue (e.g., the reports above) and with higher aerobic metabolism,^{13,102} the authors reasonably theorized that their cancerous specimens: 1. May have been substrate limited (thus limiting their aerobic metabolism), and/or 2. May have been glycolytic, which can increase the cellular concentration of reduced nicotinamide adenine dinucleotide phosphate (NADPH) relative to NADH, which may in turn cause a higher redox ratio in the tumor tissue.⁵⁸ (NADH and NADPH serve different cellular roles and are optically indistinguishable. Therefore the term “NAD(P)H” is sometimes used to indicate that both forms will be included in the optical signal. Usually the mitochondrial NADH signal will dominate, but under conditions that significantly increase NADPH relative to NADH, the redox ratio and results may be affected accordingly.^{49,89,124}) Finally, in fresh colonic biopsy tissues, the redox ratio (as $NADH/FAD$) was higher in pre-cancer and cancer tissues than in normal tissues.¹²³

Intrinsic Signals and Tissue Processing Considerations

Some evidence suggests that (often harsh) tissue post-processing procedures (with or without freezing, fixation, *etc.*) may significantly affect how intrinsic fluorescent signals respond under TPM. The studies above have obtained SHG signals from fresh (live tissue), fresh frozen, and formalin fixed biopsy tissue, and we have obtained SHG signal from both unstained and H&E stained paraffin embedded human archival breast cancer tissue (data not shown), indicating that SHG signals can be obtained from tissue subject to a range of processing techniques. On the other hand, in collagen gels we have observed qualitative differences in the collagen SHG signal in paraformaldehyde fixed vs. unfixed gels (data not shown), and others have observed that SHG signal may be diminished or absent under some fixation and histological processing/staining conditions.⁹⁰ These effects might result from tissue dehydration and shrinkage, from fixation-induced protein cross-linking which could vary with fixative type, from stains interfering with the SHG signal, or from other yet unknown sources.^{77,82} Along similar lines, in normal human lung that had been frozen and thawed, one group observed strong SHG and auto-fluorescence from fibrous connective tissue (presumably collagen) around alveolar septae at 780 nm excitation.¹¹¹ Another group using fresh mouse lung found a similar pattern at 860 nm excitation, but not at 780 nm.⁸⁶ These findings suggest that either species differences or tissue processing differences (e.g., fresh vs. frozen) may result in spectral shifting, deterioration, or physical rearrangement of some intrinsic signals. On the other hand, in their working mouse models Conklin *et al.*²¹ found that compared to unfixed live specimens, fixation, paraffin embedding, and other slide processing did not significantly impact the fluorescence properties of—nor their ability to obtain useful information from—NADH and FAD measurements. As a whole, it is evident that work is still needed in this area to further clarify whether and how fixation and different

histological processing affects TPM SHG and intrinsic fluorophore signals, and if so, what the implications are for how we compare and contrast SHG cancer studies in clinical tissue.

TPM in Human Subjects

In many cases, the goal of exploratory work in biopsies is to ultimately translate those findings toward development of *in vivo* cancer diagnostic tools. Accordingly, feasibility studies have been performed and work is ongoing for TPM cancer detection in two main areas: 1. *in vivo* TPM imaging of human skin, and 2. *in vivo* TPM endoscopy of deeper tissue areas. Each will be discussed in turn. It is relevant to note that *confocal* (one-photon) human *in vivo* skin and endoscopy imaging, and technology commercialization (e.g., Lucid's Vivascope and Mauna Kea Technology's Cellvizio or Pentax/Optiscan's ISC-1000 units, respectively), has generally run ahead of two-photon based development of similar technologies, and some comparisons of confocal vs. TPM approaches for clinical *in vivo* imaging have been presented.^{52,57,59,73,78} While discussion of these *confocal* technologies is beyond this review, at minimum they help demonstrate that clinical devices using 2P laser excitation should be achievable, especially with continued development of miniaturized lenses and 2P compatible delivery fibers.^{32,35}

in vivo TPM Imaging of Human Skin

Normal and diseased human skin has been subject to extensive analysis by TPM, both *ex vivo* and *in vivo*, for the purposes of identifying optical biopsy characteristics that might lead to less-invasive discrimination between normal and diseased tissues.^{7,15,29,45,59,62,108} Moreover, studies by several groups that have examined *ex vivo* human skin biopsies demonstrate that TPM approaches can histopathologically characterize and discriminate normal vs. cancerous skin tissues (reviewed in Tsai *et al.*,¹⁰⁸ Lin *et al.*,⁶⁸ and Paoli *et al.*⁸⁴). To avoid redundancy, we briefly highlight a few recent studies demonstrating TPM's potential for identifying skin cancer *in vivo*, as well as an emerging TPM method for skin cancer diagnosis.

Dimitrow *et al.*²⁷ used TPM of intrinsic TPEF to examine the pigmented skin cancer melanoma in 83 patients, both *ex vivo* and *in vivo*. A six-axes diagnostic matrix was constructed to distinguish between nevi (clinically benign, sharply circumscribed, chronic, usually pigmented lesions of the skin) and melanoma, with the following four features being most indicative of melanoma: 1. Overall chaotic epidermal structure; 2. Presence of dendritic cells; 3. Presence of pleomorphic cells; and 4. Poorly defined keratinocyte cell borders. Performing logistic regression analysis showed that using these criteria allowed for 85% accuracy (i.e., correct classification of the lesion) *in vivo*, and 97% accuracy *ex vivo*. This matrix also achieved an *in vivo* diagnostic sensitivity of 75%, and an *in vivo* specificity of 80%. Moreover and importantly, other elegant studies have demonstrated the potential of TPM FLIM to differentiate healthy from cancerous skin tissues.^{23,26}

Other recent exciting developments in cancer TPM imaging have used a multiphoton technique sometimes broadly referred to as "pump-probe imaging." Briefly, rather than irradiate the sample with a single near-IR pulsed laser wavelength as is done in standard TPEF and SHG TPM approaches, pump-probe imaging irradiates the sample with two different near-IR wavelengths—the pump and probe pulses respectively—which are spatially colocalized but temporally offset (by ~femtosecond delays) at the sample. The target molecules are first excited by the pump laser, after which absorption of the subsequent probe laser will depend on excited state absorption, stimulated emission, and ground state depletion effects, which in turn will vary with the pump-probe wavelengths and pump-probe delay as they interact with the molecular characteristics of the targets.^{88,117} Therefore varying the pump and probe wavelengths and the interpulse times between the pump and

probe beams, and monitoring the response signals, allows researchers to gather much more detailed molecular signatures than is available from standard TPEF techniques. Using such techniques, researchers have been able optically distinguish eumelanin from pheomelanin, despite their otherwise very similar linear near-IR absorptive properties.⁸⁸ Going further, the same group then microscopically differentiated and measured eumelanin and pheomelanin content in *ex vivo* human skin biopsies, and found that the ratio of eumelanin to pheomelanin could, along with other diagnostic criteria, help distinguish between melanoma, dysplastic nevi, and benign nevi.⁷⁵ These studies, also demonstrated *in vivo* in a mouse model,⁷⁶ offer exciting evidence that such pump-probe techniques, perhaps with the continued discovery of additional pump-probe molecular signatures in cancer tissue, may ultimately offer highly accurate *in vivo* diagnostics for skin cancer.

in vivo Two-Photon Microendoscopy

In addition to *in vivo* skin imaging, another clinical TPM approach generating significant attention for cancer diagnostics is focused on developing technologies for intracavitary or intracorporeal two-photon microendoscopy (TPME). The “penetration depth” of normal TPM imaging is technically restricted to, at best, within ~1 mm of the imaged surface. Therefore for TPM cancer imaging of areas besides skin, microendoscopic approaches are required. Generally speaking, two photon microendoscopic approaches can be grouped into two broad categories: 1. Those using rigid, needle-like lenses, for imaging nearer the body surface (with keyhole incisions, for example), and 2. Those using these or similar lenses attached to a flexible fiberoptic probe, thus potentially enabling much deeper intracavity imaging, in traditional “endoscopic” fashion. Ideally such tools—through either surgical or non-surgical intracavitary access— might eliminate the need for more invasive or surgical biopsy-based approaches to cancer detection. Moreover, such approaches may eventually lead further to targeted microsurgical or ablation approaches based on non-linear optical microscopy.⁴⁰

Toward these goals, considerable effort has been devoted to developing and improving TPME technologies. Yet contrary to the relative abundance of human *in vivo* work with 1P confocal microendoscopy, there have been relatively few human TPME studies, and fewer still pertaining specifically to cancer. Presumably this is because TPME technology is more nascent, partly because confocal microendoscopy technologies are not directly transferable due to TPME’s requirement for fiberoptics in which 2P pulses are not degraded.⁵² However the advent of photonic crystal fibers and pre-chirped multicore fibers have advanced the technology in this regard, and miniaturization of imaging lenses (e.g., gradient index (GRIN) lenses) and scanning systems have further facilitated development fiber-based TPME systems toward clinical use.^{32,35,52,57,61,64,66,78} In fact, there have been numerous demonstrations of fiber-based TPME of intact living tissue (e.g., Rivera *et al.*⁹⁶, and references discussed therein), indicating that the fiber-based limitations for TPME may have been largely overcome. These advances, combined with the fact that fiber based *confocal* microendoscopy is now a relatively routine clinical procedure,⁸⁵ suggest that further refinement of prototype fiber-based TPME devices and navigating the important regulatory and safety considerations are the most likely next steps for advancing such “flexible fiber-type” TPME devices to clinical trial.

Human *in vivo* demonstrations of TPME have to our knowledge thus far used rigid needle-like endoscopic GRIN lenses to image SHG in muscle sarcomeres right below the skin surface,⁶⁹ and to image ulcerative skin wounds.⁶¹ Cancer related pre-clinical *in vivo* animal work has also used fiber-based TPME to identify pre-cancerous goblet cells in mouse intestine,² and other work has explored imaging *in vivo* and *ex vivo* tissue with newly available “stick” objective lenses, which suffer from less spherical aberration than GRIN lenses, and have the potential to image laparoscopically up to centimeters below the body

surface.^{99,115} Finally, in two slightly different but exciting TPME applications for cancer diagnostics and treatment, non-linear near-IR based laser microsurgery enables selective ablation of gold nanorod targeted cells,^{40,48} and TPME flow cytometry methods may eventually enable *in vivo* identification of circulating tumor cells.^{14,46} With these and similar ongoing studies, and with continued miniaturization and “fiberization” of TPME technology as discussed above, intracorporeal and intracavitary demonstrations of TPME specifically for human cancer should soon follow.

Clinical and Pre-Clinical TPM Instrumentation

Several commercialized TPM devices further demonstrate the potential for TPM skin imaging in the clinic, including JenLab’s DermaInspect and MPTFlex clinically oriented MPM imagers (Jenlab GmbH, Saarbrücken, Germany). Many of the above skin studies were performed with these or very similar instruments. Based on the published reports above, these units scan at ~.05–1 frames/second dependent on the scan area and pixel resolution (and see data presented on Jenlab’s website), which is typical for standard TPM scanning configurations, and precludes realtime imaging at video rates which ultimately may be useful for cancer diagnostics *in vivo*, especially for monitoring physiologic activity. However, faster commercial confocal units (e.g., Lucid Vivascope, 9 frames/sat ~1K 9 1 K), and demonstrations of several TPM instruments that can achieve video-rate image acquisition *in vivo* (for some frame sizes),^{57,65} suggest that improved acquisition speed should be within reach of evolving clinical TPM technologies.

To our knowledge a flexible fiber TPM clinical microendoscope has not yet seen commercialization. However, in an intriguing potential pre-commercial development, Lelek *et al.*⁶⁶ have performed “proof of concept” imaging of fluorescently labeled human colon crypt cells (apparently *ex vivo*), using a commercial CellVizio confocal microendoscope adapted to TPME by addition of a Ti:Sapphire laser and multicore fiber with pulse precompensation. The authors suggest that this TPME design confers ultimately higher frame rates compared to single core fiber based TPME systems.⁶⁶ Thus, it is feasible that commercial development of a flexible fiber based TPME may occur within the relatively near future.

Safety Considerations

Two-photon microscopy of human skin can cause thermal mechanical damage at the epidermal-dermal junction, which is believed to result from linear (one photon) absorption of the near-IR irradiation by melanin, and can be at least partially mitigated by engineering controls to reduce the pulse repetition rate at the sample (thus lowering average power while maintaining peak power).⁷⁴ On the one hand, based on some estimates,³¹ the laser powers and scan rates as described in most of the *in vivo/ex vivo* skin studies discussed above are not likely to incur more damage than one might get from natural UV exposure. On the other hand, the subject’s total exposure to and potential for damage from near-IR irradiation will likely depend on many factors including average and peak laser powers used during a session; total duration of exposure as influenced by scan rates and total number of XY planes or sections taken; and perhaps frequency or repetition of exposures to the same skin area over months or years. Therefore, further investigation and standardization of these criteria as they impact potential tissue damage will be important as clinical development and commercialization of new devices moves forward, so that appropriate risk assessments can be made regarding clinical safety. As discussed above, *in vivo* instruments that can maximize image acquisition speeds while minimizing total irradiation power (i.e., maximizing sensitivity) may help mitigate some of these concerns, while also being best able to minimize motion artifacts and capture physiology in real-time. Dela Cruz *et al.*²⁴ present an informed discussion of many of these issues, and demonstrate that in at least

some tissue types, images of suitable S/N and quality can be obtained at non-mutagenic laser doses. Thus while more work in these areas is needed, at present it appears that TPM technologies for clinical imaging will likely have risk profiles no worse than (and perhaps better than) many current clinical imaging modalities (X-ray, CT scan, etc.).

FUTURE DIRECTIONS

Many other investigations underway apply TPM to human imaging and diseases beyond cancer, including other skin diseases, eye imaging, vascular and cardiovascular imaging, brain imaging, drug delivery, tissue engineering, fibrosis of liver or other organs, muscle disease, and other organ diseases for which TPM may provide diagnostic value.^{9,28,59,60,110} Other TPM-related advanced techniques are being developed which include coherent anti-Stokes Raman scattering (CARS) imaging,^{35,79,80} and development of SHG-generating exogenous dyes and nanoprobe for *in vivo* imaging of cellular events.^{83,95} These and other developing TPM applications, together with the ongoing progress in human *in vivo* TPM cancer imaging discussed herein, will advance our ability to non-invasively diagnose and treat cancer as we progress into the coming decades.

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REFERENCES

1. Bahlmann K, So PT, Kirber M, Reich R, Kosicki B, McGonagle W, Bellve K. Multifocal multiphoton microscopy (MMM) at a frame rate beyond 600 Hz. *Opt. Express*. 2007; 15:10991–10998. [PubMed: 19547456]
2. Bao H, Boussioutas A, Reynolds J, Russell S, Gu M. Imaging of goblet cells as a marker for intestinal metaplasia of the stomach by one-photon and two-photon fluorescence endomicroscopy. *J. Biomed. Opt.* 2009; 14:064031. [PubMed: 20059269]
3. Bastiaens PI, Squire A. Fluorescence lifetime imaging microscopy: spatial resolution of biochemical processes in the cell. *Trends Cell Biol.* 1999; 9:48–52. [PubMed: 10087617]
4. Beerling E, Ritsma L, Vrisekoop N, Derksen PW, van Rheenen J. Intravital microscopy: new insights into metastasis of tumors. *J. Cell Sci.* 2011; 124:299–310. [PubMed: 21242309]
5. Bewersdorf J, Egner A, Hell SW. Multifocal Multi-Photon Microscopy. In: Pawley, JB., editor. *Handbook of Biological Confocal Microscopy*. Springer; New York: 2006. p. 550-560.
6. Bewersdorf J, Pick R, Hell SW. Multifocal multiphoton microscopy. *Opt. Lett.* 1998; 23:655–657. [PubMed: 18087301]
7. Breunig HG, Studier H, König K. Multiphoton excitation characteristics of cellular fluorophores of human skin *in vivo*. *Opt. Express*. 2010; 18:7857–7871. [PubMed: 20588627]
8. Brown E, McKee T, diTomaso E, Pluen A, Seed B, Boucher Y, Jain RK. Dynamic imaging of collagen and its modulation in tumors *in vivo* using second-harmonic generation. *Nat. Med.* 2003; 9:796–800. [PubMed: 12754503]
9. Campagnola P. Second harmonic generation imaging microscopy: applications to diseases diagnostics. *Anal. Chem.* 2011; 83:3224–3231. [PubMed: 21446646]
10. Campagnola PJ, Loew LM. Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. *Nat. Biotechnol.* 2003; 21:1356–1360. [PubMed: 14595363]
11. Campagnola PJ, Millard AC, Terasaki M, Hoppe PE, Malone CJ, Mohler WA. Three-dimensional high-resolution second-harmonic generation imaging of endogenous structural proteins in biological tissues. *Biophys. J.* 2002; 82:493–508. [PubMed: 11751336]

12. Campagnola PJ, Wei MD, Lewis A, Loew LM. High-resolution nonlinear optical imaging of live cells by second harmonic generation. *Biophys. J.* 1999; 77:3341–3349. [PubMed: 10585956]
13. Chance B, Schoener B, Oshino R, Itshak F, Nakase Y. Oxidation–reduction ratio studies of mitochondria in freeze-trapped samples. NADH and flavoprotein fluorescence signals. *J. Biol. Chem.* 1979; 254:4764–4771. [PubMed: 220260]
14. Chang YC, Ye JY, Thomas TP, Cao Z, Kotlyar A, Tkaczyk ER, Baker JR Jr, Norris TB. Fiberoptic multiphoton flow cytometry in whole blood and in vivo. *J. Biomed. Opt.* 2010; 15:047004. [PubMed: 20799835]
15. Chen J, Lee A, Zhao J, Wang H, Lui H, McLean DI, Zeng H. Spectroscopic characterization and microscopic imaging of extracted and in situ cutaneous collagen and elastic tissue components under two-photon excitation. *Skin. Res. Technol.* 2009; 15:418–426. [PubMed: 19832952]
16. Chen Y, Periasamy A. Characterization of two-photon excitation fluorescence lifetime imaging microscopy for protein localization. *Microsc. Res. Tech.* 2004; 63:72–80. [PubMed: 14677136]
17. Chen J, Zhuo S, Chen G, Yan J, Yang H, Liu N, Zheng L, Jiang X, Xie S. Establishing diagnostic features for identifying the mucosa and submucosa of normal and cancerous gastric tissues by multiphoton microscopy. *Gastrointest. Endosc.* 2011; 73:802–807. [PubMed: 21457819]
18. Cicchi R, Crisci A, Cosci A, Nesi G, Kapsokalyvas D, Giancane S, Carini M, Pavone FS. Time- and spectral-resolved two-photon imaging of healthy bladder mucosa and carcinoma in situ. *Opt. Express.* 2010; 18:3840–3849. [PubMed: 20389394]
19. Condeelis J, Segall JE. Intravital imaging of cell movement in tumours. *Nat. Rev. Cancer.* 2003; 3:921–930. [PubMed: 14737122]
20. Conklin MW, Eickhoff JC, Riching KM, Pehlke CA, Eliceiri KW, Provenzano PP, Friedl A, Keely PJ. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am. J. Pathol.* 2011; 178:1221–1232. [PubMed: 21356373]
21. Conklin MW, Provenzano PP, Eliceiri KW, Sullivan R, Keely PJ. Fluorescence lifetime imaging of endogenous fluorophores in histopathology sections reveals differences between normal and tumor epithelium in carcinoma in situ of the breast. *Cell Biochem. Biophys.* 2009; 53:145–157. [PubMed: 19259625]
22. Cox G, Kable E, Jones A, Fraser I, Manconi F, Gorrell MD. 3-dimensional imaging of collagen using second harmonic generation. *J. Struct. Biol.* 2003; 141:53–62. [PubMed: 12576020]
23. De Giorgi V, Massi S, Sestini S, Cicchi R, Pavone FS, Lotti T. Combined non-linear laser imaging (two-photon excitation fluorescence microscopy, fluorescence lifetime imaging microscopy, multispectral multiphoton microscopy) in cutaneous tumours: first experiences. *J. Eur. Acad. Dermatol. Venereol. (JEADV).* 2009; 23:314–316.
24. Dela Cruz JM, McMullen JD, Williams RM, Zipfel WR. Feasibility of using multiphoton excited tissue autofluorescence for in vivo human histopathology. *Biomed. Opt. Express.* 2010; 1:1320–1330. [PubMed: 21258552]
25. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. *Science.* 1990; 248:73–76. [PubMed: 2321027]
26. Dimitrow E, Riemann I, Ehlers A, Koehler MJ, Norgauer J, Elsner P, König K, Kaatz M. Spectral fluorescence lifetime detection and selective melanin imaging by multiphoton laser tomography for melanoma diagnosis. *Exp. Dermatol.* 2009; 18:509–515. [PubMed: 19243426]
27. Dimitrow E, Ziemer M, Koehler MJ, Norgauer J, König K, Elsner P, Kaatz M. Sensitivity and specificity of multiphoton laser tomography for in vivo and ex vivo diagnosis of malignant melanoma. *J. Invest. Dermatol.* 2009; 129:1752–1758. [PubMed: 19177136]
28. Dong CY, Campagnola PJ. Optical diagnostics of tissue pathology by multiphoton microscopy. *Expert Opin. Med. Diag.* 2010; 4:519–529.
29. Ericson MB, Simonsson C, Guldbrand S, Ljungblad C, Paoli J, Smedh M. Two-photon laser-scanning fluorescence microscopy applied for studies of human skin. *J. Biophotonics.* 2008; 1:320–330. [PubMed: 19343655]
30. Fine S, Hansen WP. Optical second harmonic generation in biological systems. *Appl. Opt.* 1971; 10:2350–2353. [PubMed: 20111328]

31. Fischer F, Volkmer B, Puschmann S, Greinert R, Breitbart W, Kiefer J, Wepf R. Risk estimation of skin damage due to ultrashort pulsed, focused near-infrared laser irradiation at 800 nm. *J. Biomed. Opt.* 2008; 13:041320. [PubMed: 19021328]
32. Flusberg BA, Cocker ED, Piyawattanametha W, Jung JC, Cheung EL, Schnitzer MJ. Fiber-optic fluorescence imaging. *Nat. Methods.* 2005; 2:941–950. [PubMed: 16299479]
33. Freund I, Deutsch M. Macroscopic polarity of connective tissue is due to discrete polar structures. *Biopolymers.* 1986; 25:601–606. [PubMed: 3708106]
34. Fricke M, Nielsen T. Two-dimensional imaging without scanning by multifocal multiphoton microscopy. *Appl. Opt.* 2005; 44:2984–2988. [PubMed: 15929287]
35. Fu L, Gu M. Fibre-optic nonlinear optical microscopy and endoscopy. *J. Microsc.* 2007; 226:195–206. [PubMed: 17535259]
36. Fukumura D, Duda DG, Munn LL, Jain RK. Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models. *Microcirculation.* 2010; 17:206–225. [PubMed: 20374484]
37. Ammer, A. Gatesman; Hayes, KE.; Martin, KH.; Zhang, L.; Spirou, GA.; Weed, SA. Multi-photon imaging of tumor cell invasion in an orthotopic mouse model of oral squamous cell carcinoma. *J. Vis. Exp.* 2011; 53:e2941.
38. Ghajar CM, Bissell MJ. Extracellular matrix control of mammary gland morphogenesis and tumorigenesis: insights from imaging. *Histochem. Cell Biol.* 2008; 130:1105–1118. [PubMed: 19009245]
39. Goppert-Meyer M. Uber Elementarakte mit zwei Quantensprungen. Gottinger Dissertation. *Ann. Phys.* 1931; 9:273–294.
40. Gu M, Bao HC, Li JL. Cancer-cell microsurgery using nonlinear optical endomicroscopy. *J. Biomed. Opt.* 2010; 15:050502. [PubMed: 21054074]
41. Guo Y, Ho PP, Tirkslunas A, Liu F, Alfano RR. Optical harmonic generation from animal tissues by the use of picosecond and femtosecond laser pulses. *Appl. Opt.* 1996; 35:6810–6813. [PubMed: 21151266]
42. Guo Y, Savage HE, Liu F, Schantz SP, Ho PP, Alfano RR. Subsurface tumor progression investigated by noninvasive optical second harmonic tomography. *Proc. Natl Acad. Sci. USA.* 1999; 96:10854–10856. [PubMed: 10485915]
43. Han X, Brown E. Measurement of the ratio of forward-propagating to back-propagating second harmonic signal using a single objective. *Opt. Express.* 2010; 18:10538–10550. [PubMed: 20588906]
44. Han X, Burke RM, Zettel ML, Tang P, Brown EB. Second harmonic properties of tumor collagen: determining the structural relationship between reactive stroma and healthy stroma. *Opt. Express.* 2008; 16:1846–1859. [PubMed: 18542263]
45. Hanson KM, Bardeen CJ. Application of non-linear optical microscopy for imaging skin. *Photochem. Photobiol.* 2009; 85:33–44. [PubMed: 19161396]
46. He W, Wang H, Hartmann LC, Cheng JX, Low PS. In vivo quantitation of rare circulating tumor cells by multiphoton intravital flow cytometry. *Proc. Natl Acad. Sci. USA.* 2007; 104:11760–11765. [PubMed: 17601776]
47. Hompland T, Erikson A, Lindgren M, Lindmo T, de Lange Davies C. Second-harmonic generation in collagen as a potential cancer diagnostic parameter. *J. Biomed. Opt.* 2008; 13:054050. [PubMed: 19021430]
48. Huang X, El-Sayed IH, Qian W, El-Sayed MA. Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods. *J. Am. Chem. Soc.* 2006; 128:2115–2120. [PubMed: 16464114]
49. Huang S, Heikal AA, Webb WW. Two-photon fluorescence spectroscopy and microscopy of NAD(P)H and flavoprotein. *Biophys. J.* 2002; 82:2811–2825. [PubMed: 11964266]
50. Huang Z, Li Z, Chen R, Lin J, Li Y, Li C. In vitro imaging of thyroid tissues using two-photon excited fluorescence and second harmonic generation. *Photomed. Laser Surg.* 2010; 28(Suppl 1):S129–S133. [PubMed: 20649422]

51. Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev. Dyn.* 2006; 235:3222–3229. [PubMed: 17029292]
52. Jung JC, Schnitzer MJ. Multiphoton endoscopy. *Opt. Lett.* 2003; 28:902–904. [PubMed: 12816240]
53. Kaiser W, Garret C. Two-photon excitation in CaF₂: Eu²⁺ Phys. Rev. Lett. 1961; 7:229–231.
54. Kedrin D, Wyckoff J, Sahai E, Condeelis J, Segall JE. Imaging tumor cell movement in vivo. *Curr. Protoc. Cell Biol.* 2007 Chapter 19:Unit 19.17.
55. Kim KH, Buehler C, Bahlmann K, Ragan T, Lee WC, Nedivi E, Heffer EL, Fantini S, So PT. Multifocal multiphoton microscopy based on multianode photomultiplier tubes. *Opt. Express.* 2007; 15:11658–11678. [PubMed: 19547526]
56. Kim BM, Eichler J, Reiser KM, Rubenchik AM, Da Silva LB. Collagen structure and nonlinear susceptibility: effects of heat, glycation, and enzymatic cleavage on second harmonic signal intensity. *Lasers Surg. Med.* 2000; 27:329–335. [PubMed: 11074509]
57. Kim P, Puoris'haag M, Cote D, Lin CP, Yun SH. In vivo confocal and multiphoton microendoscopy. *J. Biomed. Opt.* 2008; 13:010501. [PubMed: 18315346]
58. Kirkpatrick ND, Brewer MA, Utzinger U. Endogenous optical biomarkers of ovarian cancer evaluated with multiphoton microscopy. *Cancer Epidemiol. Biomarkers Prev.* 2007; 16:2048–2057. [PubMed: 17932352]
59. Koehler MJ, Speicher M, Lange-Asschenfeldt S, Stockfleth E, Metz S, Elsner P, Kaatz M, Konig K. Clinical application of multiphoton tomography in combination with confocal laser scanning microscopy for in vivo evaluation of skin diseases. *Exp. Dermatol.* 2011; 20:589–594. [PubMed: 21539618]
60. Konig K. Multiphoton microscopy in life sciences. *J. Microsc.* 2000; 200:83–104. [PubMed: 11106949]
61. Konig, K.; Buckle, R.; Weinigel, M.; Elsner, P.; Kaatz, M. Clinical multiphoton tomography and clinical two-photon microendoscopy. In: Periasamy, A.; So, PT., editors. *Multiphoton Microscopy in the Biomedical Sciences IX*, Proceedings of the SPIE; New York: Oxford University Press; 2009. p. 7183191-7183199.
62. Konig K, Speicher M, Buckle R, Reckfort J, McKenzie G, Welzel J, Koehler MJ, Elsner P, Kaatz M. Clinical optical coherence tomography combined with multiphoton tomography of patients with skin diseases. *J. Biophotonics.* 2009; 2:389–397. [PubMed: 19598177]
63. Le Devedec SE, van Roosmalen W, Pont C, Lalai R, de Bont H, van de Water B. Two-photon intravital multicolour imaging to study metastatic behaviour of cancer cells in vivo. *Methods Mol. Biol.* 2011; 769:331–349. [PubMed: 21748686]
64. Le Harzic R, Riemann I, Weinigel M, Konig K, Messerschmidt B. Rigid and high-numerical-aperture two-photon fluorescence endoscope. *Appl. Opt.* 2009; 48:3396–3400. [PubMed: 19543347]
65. Lee AMD, Wang H, Yu Y, Tang S, Zhao J, Lui H, McLean DI, Zeng H. In vivo video rate multiphoton microscopy imaging of human skin. *Opt. Lett.* 2011; 36:2865–2867. [PubMed: 21808340]
66. Lelek M, Suran E, Louradour F, Barthelemy A, Viellerobe B, Lacombe F. Coherent femtosecond pulse shaping for the optimization of a non-linear micro-endoscope. *Opt. Express.* 2007; 15:10154–10162. [PubMed: 19547364]
67. Lin HJ, Herman P, Lakowicz JR. Fluorescence lifetime-resolved pH imaging of living cells. *Cytometry A: J. Int. Soc. Anal. Cytol.* 2003; 52:77–89.
68. Lin SJ, Jee SH, Dong CY. Multiphoton microscopy: a new paradigm in dermatological imaging. *Eur. J. Dermatol.* 2007; 17:361–366. [PubMed: 17673377]
69. Llewellyn ME, Barretto RP, Delp SL, Schnitzer MJ. Minimally invasive high-speed imaging of sarcomere contractile dynamics in mice and humans. *Nature.* 2008; 454:784–788. [PubMed: 18600262]
70. Lohela M, Werb Z. Intravital imaging of stromal cell dynamics in tumors. *Curr. Opin. Genet. Dev.* 2010; 20:72–78. [PubMed: 19942428]
71. Lunt SJ, Gray C, Reyes-Aldasoro CC, Matcher SJ, Tozer GM. Application of intravital microscopy in studies of tumor microcirculation. *J. Biomed. Opt.* 2010; 15:011113. [PubMed: 20210439]

72. Martini J, Andresen V, Anselmetti D. Scattering suppression and confocal detection in multifocal multi-photon microscopy. *J. Biomed. Opt.* 2007; 12:034010. [PubMed: 17614718]
73. Masters B, So P. Confocal microscopy and multi-photon excitation microscopy of human skin in vivo. *Opt. Express.* 2001; 8:2–10. [PubMed: 19417779]
74. Masters BR, So PT, Buehler C, Barry N, Sutin JD, Mantulin WW, Gratton E. Mitigating thermal mechanical damage potential during two-photon dermal imaging. *J. Biomed. Opt.* 2004; 9:1265–1270. [PubMed: 15568947]
75. Matthews TE, Piletic IR, Selim MA, Simpson MJ, Warren WS. Pump-probe imaging differentiates melanoma from melanocytic nevi. *Sci. Transl. Med.* 2011; 3:71–72.
76. Matthews TE, Wilson JW, Degan S, Simpson MJ, Jin JY, Zhang JY, Warren WS. In vivo and ex vivo epi-mode pump-probe imaging of melanin and microvasculature. *Biomed Opt. Express.* 2011; 2:1576–1583. [PubMed: 21698020]
77. Meek KM. The use of glutaraldehyde and tannic acid to preserve reconstituted collagen for electron microscopy. *Histochemistry.* 1981; 73:115–120. [PubMed: 6797991]
78. Meier R, Kromer K, Stepp H, Sroka R. A comparison of confocal and two-photon microendoscopy. *IFMBE Proc.* 2009; 25(6):177–178.
79. Meyer T, Bergner N, Bielecki C, Krafft C, Akimov D, Romeike BF, Reichart R, Kalff R, Dietzek B, Popp J. Nonlinear microscopy, infrared, and Raman microspectroscopy for brain tumor analysis. *J. Biomed. Opt.* 2011; 16:021113. [PubMed: 21361676]
80. Min W, Freudiger CW, Lu S, Xie XS. Coherent nonlinear optical imaging: beyond fluorescence microscopy. *Annu. Rev. Phys. Chem.* 2011; 62:507–530. [PubMed: 21453061]
81. Mohler W, Millard AC, Campagnola PJ. Second harmonic generation imaging of endogenous structural proteins. *Methods.* 2003; 29:97–109. [PubMed: 12543075]
82. Nadiarnykh O, LaComb RB, Brewer MA, Campagnola PJ. Alterations of the extracellular matrix in ovarian cancer studied by Second Harmonic Generation imaging microscopy. *BMC Cancer.* 2010; 10:94. [PubMed: 20222963]
83. Pantazis P, Maloney J, Wu D, Fraser SE. Second harmonic generating (SHG) nanoprobe for in vivo imaging. *Proc. Natl Acad. Sci. USA.* 2010; 107:14535–14540. [PubMed: 20668245]
84. Paoli J, Smedh M, Ericson MB. Multiphoton laser scanning microscopy—a novel diagnostic method for superficial skin cancers. *Semin. Cutan. Med. Surg.* 2009; 28:190–195. [PubMed: 19782943]
85. Paull PE, Hyatt BJ, Wassef W, Fischer AH. Confocal laser endomicroscopy: a primer for pathologists. *Arch. Pathol. Lab. Med.* 2011; 135:1343–1348. [PubMed: 21970490]
86. Pavlova I, Hume KR, Yazinski SA, Peters RM, Weiss RS, Webb WW. Multiphoton microscopy as a diagnostic imaging modality for lung cancer. *Proc. Soc. Photo Opt. Instrum. Eng.* 2010; 7569:756918. [PubMed: 20445820]
87. Pawley, JB., editor. *Handbook of Biological Confocal Microscopy.* Springer; New York: 2006. p. 1016
88. Piletic IR, Matthews TE, Warren WS. Probing near-infrared photorelaxation pathways in eumelanins and pheomelanins. *J. Phys. Chem. A.* 2010; 114:11483–11491. [PubMed: 20882951]
89. Piston DW, Masters BR, Webb WW. Three-dimensionally resolved NAD(P)H cellular metabolic redox imaging of the in situ cornea with two-photon excitation laser scanning microscopy. *J. Microsc.* 1995; 178:20–27. [PubMed: 7745599]
90. Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med.* 2006; 4:38. [PubMed: 17190588]
91. Provenzano PP, Eliceiri KW, Keely PJ. Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. *Clin. Exp. Metastasis.* 2009; 26:357–370. [PubMed: 18766302]
92. Provenzano PP, Eliceiri KW, Yan L, Ada-Nguema A, Conklin MW, Inman DR, Keely PJ. Nonlinear optical imaging of cellular processes in breast cancer. *Microsc. Microanal.* 2008; 14:532–548. [PubMed: 18986607]

93. Provenzano PP, Inman DR, Eliceiri KW, Trier SM, Keely PJ. Contact guidance mediated three-dimensional cell migration is regulated by Rho/ROCK-dependent matrix reorganization. *Biophys. J.* 2008; 95:5374–5384. [PubMed: 18775961]
94. Provenzano PP, Rueden CT, Trier SM, Yan L, Ponik SM, Inman DR, Keely PJ, Eliceiri KW. Nonlinear optical imaging and spectral-lifetime computational analysis of endogenous and exogenous fluorophores in breast cancer. *J. Biomed. Opt.* 2008; 13:031220. [PubMed: 18601544]
95. Reeve JE, Anderson HL, Clays K. Dyes for biological second harmonic generation imaging. *Phys. Chem. Chem. Phys.* 2010; 12:13484–13498. [PubMed: 20820473]
96. Rivera DR, Brown CM, Ouzounov DG, Pavlova I, Kobat D, Webb WW, Xu C. Compact and flexible raster scanning multiphoton endoscope capable of imaging unstained tissue. *Proc. Natl Acad. Sci. USA.* 2011; 108:17598–17603. [PubMed: 22006303]
97. Roberts MS, Dancik Y, Prow TW, Thorling CA, Lin LL, Grice JE, Robertson TA, Konig K, Becker W. Non-invasive imaging of skin physiology and percutaneous penetration using fluorescence spectral and lifetime imaging with multiphoton and confocal microscopy. *Eur. J. Pharm. Biopharm.* 2011; 77:469–488. [PubMed: 21256962]
98. Rocheleau JV, Piston DW. Two-photon excitation microscopy for the study of living cells and tissues. *Curr. Protoc. Cell Biol.* 2003 Chapter 4:Unit 4.11.
99. Rogart JN, Nagata J, Loeser CS, Roorda RD, Aslanian H, Robert ME, Zipfel WR, Nathanson MH. Multiphoton imaging can be used for microscopic examination of intact human gastrointestinal mucosa ex vivo. *Clin. Gastroenterol. Hepatol.* 2008; 6:95–101. [PubMed: 18065276]
100. Roth S, Freund I. Second harmonic generation in collagen. *J. Chem. Phys.* 1979; 70:1637.
101. Schedin P, Keely PJ. Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression. *Cold Spring Harb. Perspect. Biol.* 2011; 3:a003228. [PubMed: 20980442]
102. Skala MC, Riching KM, Gendron-Fitzpatrick A, Eickhoff J, Eliceiri KW, White JG, Ramanujam N. In vivo multiphoton microscopy of NADH and FAD redox states, fluorescence lifetimes, and cellular morphology in precancerous epithelia. *Proc. Natl Acad. Sci. USA.* 2007; 104:19494–19499. [PubMed: 18042710]
103. Stoller P, Celliers PM, Reiser KM, Rubenchik AM. Quantitative second-harmonic generation microscopy in collagen. *Appl. Opt.* 2003; 42:5209–5219. [PubMed: 12962402]
104. Straub M, Lodemann P, Holroyd P, Jahn R, Hell SW. Live cell imaging by multifocal multiphoton microscopy. *Eur. J. Cell Biol.* 2000; 79:726–734. [PubMed: 11089921]
105. Tewari AK, Shevchuk MM, Sterling J, Grover S, Herman M, Yadav R, Mudalair K, Srivastava A, Rubin MA, Zipfel WR, Maxfield FR, Xu C, Webb WW, Mukherjee S. Multiphoton microscopy for structure identification in human prostate and periprostic tissue: implications in prostate cancer surgery. *BJU Int.* 2011; 108(9):1421–1429. [PubMed: 21443651]
106. Theodossiou T, Rapti GS, Hovhannisyan V, Georgiou E, Politopoulos K, Yova D. Thermally induced irreversible conformational changes in collagen probed by optical second harmonic generation and laser-induced fluorescence. *Lasers Med. Sci.* 2002; 17:34–41. [PubMed: 11845366]
107. Tozer GM, Ameer-Beg SM, Baker J, Barber PR, Hill SA, Hodgkiss RJ, Locke R, Prise VE, Wilson I, Vojnovic B. Intravital imaging of tumour vascular networks using multi-photon fluorescence microscopy. *Adv. Drug Deliv. Rev.* 2005; 57:135–152. [PubMed: 15518926]
108. Tsai TH, Jee SH, Dong CY, Lin SJ. Multiphoton microscopy in dermatological imaging. *J. Dermatol. Sci.* 2009; 56:1–8. [PubMed: 19699614]
109. van Munster EB, Gadella TW. Fluorescence lifetime imaging microscopy (FLIM). *Adv. Biochem. Eng. Biotechnol.* 2005; 95:143–175. [PubMed: 16080268]
110. Wang BG, Konig K, Halbhauer KJ. Two-photon microscopy of deep intravital tissues and its merits in clinical research. *J. Microsc.* 2010; 238:1–20. [PubMed: 20384833]
111. Wang CC, Li FC, Wu RJ, Hovhannisyan VA, Lin WC, Lin SJ, So PT, Dong CY. Differentiation of normal and cancerous lung tissues by multiphoton imaging. *J. Biomed. Opt.* 2009; 14:044034. [PubMed: 19725745]
112. Wang W, Wyckoff JB, Frohlich VC, Oleynikov Y, Huttelmaier S, Zavadil J, Cermak L, Bottinger EP, Singer RH, White JG, Segall JE, Condeelis JS. Single cell behavior in metastatic primary

mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Res.* 2002; 62:6278–6288. [PubMed: 12414658]

113. Wang W, Wyckoff JB, Goswami S, Wang Y, Sidani M, Segall JE, Condeelis JS. Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. *Cancer Res.* 2007; 67:3505–3511. [PubMed: 17440055]
114. Wilder-Smith P, Krasieva T, Jung WG, Zhang J, Chen Z, Osann K, Tromberg B. Noninvasive imaging of oral premalignancy and malignancy. *J. Biomed. Opt.* 2005; 10:051601. [PubMed: 16292949]
115. Williams RM, Flesken-Nikitin A, Ellenson LH, Connolly DC, Hamilton TC, Nikitin AY, Zipfel WR. Strategies for high-resolution imaging of epithelial ovarian cancer by laparoscopic nonlinear microscopy. *Transl. Oncol.* 2010; 3:181–194. [PubMed: 20563260]
116. Williams RM, Zipfel WR, Webb WW. Interpreting second-harmonic generation images of collagen I fibrils. *Biophys. J.* 2005; 88:1377–1386. [PubMed: 15533922]
117. Wilson, JW.; Matthews, TE.; Degan, S.; Zhang, JY.; Simpson, MJ.; Warren, WS. CLEO:2011—laser applications to photonic applications, OSA Technical Digest (CD). Optical Society of America; 2011. Pump-probe melanoma imaging: applications to high-resolution and in vivo microscopy. paper PDPB5, 2011
118. Wolf K, Alexander S, Schacht V, Coussens LM, von Andrian UH, van Rheenen J, Deryugina E, Friedl P. Collagen-based cell migration models in vitro and in vivo. *Semin. Cell Dev. Biol.* 2009; 20:931–941. [PubMed: 19682592]
119. Yan J, Chen G, Chen J, Liu N, Zhuo S, Yu H, Ying M. A pilot study of using multiphoton microscopy to diagnose gastric cancer. *Surg. Endosc.* 2011; 25:1425–1430. [PubMed: 21046158]
120. Yan L, Rueden CT, White JG, Eliceiri KW. Applications of combined spectral lifetime microscopy for biology. *Biotechniques.* 2006; 41:249, 251, 253. [PubMed: 16989084]
121. Zal T, Chodaczek G. Intravital imaging of anti-tumor immune response and the tumor microenvironment. *Semin. Immunopathol.* 2010; 32:305–317. [PubMed: 20652252]
122. Zhuo S, Chen J, Xie S, Hong Z, Jiang X. Extracting diagnostic stromal organization features based on intrinsic two-photon excited fluorescence and second-harmonic generation signals. *J. Biomed. Opt.* 2009; 14:020503. [PubMed: 19405709]
123. Zhuo S, Yan J, Chen G, Chen J, Liu Y, Lu J, Zhu X, Jiang X, Xie S. Label-free monitoring of colonic cancer progression using multiphoton microscopy. *Biomed. Opt. Express.* 2011; 2:615–619. [PubMed: 21412466]
124. Zipfel WR, Williams RM, Christie R, Nikitin AY, Hyman BT, Webb WW. Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. *Proc. Natl Acad. Sci. USA.* 2003; 100:7075–7080. [PubMed: 12756303]
125. Zipfel WR, Williams RM, Webb WW. Non-linear magic: multiphoton microscopy in the biosciences. *Nat. Biotechnol.* 2003; 21:1369–1377. [PubMed: 14595365]
126. Zoumi A, Yeh A, Tromberg BJ. Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence. *Proc. Natl Acad. Sci. USA.* 2002; 99:11014–11019. [PubMed: 12177437]

FIGURE 1.

Non-linear TPEF and SHG compared. Shown are the Perrin–Jablonski fluorescence diagram for TPEF (left), and the energy-level diagram for SHG (right). In TPEF, two photons of incident frequency ω_i and wavelength λ_i (in red) are simultaneously absorbed by a fluorophore, to produce a single photon (in green) whose wavelength is greater than one-half the irradiating wavelength λ_i (i.e., with some net energy loss). In SHG, two photons of frequency ω_i and wavelength λ_i (in red) interacting simultaneously with a non-centrosymmetric target, combine to form a single photon (in blue) whose wavelength is exactly half that of the incident wavelength λ_i (i.e., no net energy loss). Hence, 2P irradiation of an SHG generating target at 810 nm will emit SHG photons at 405 nm, whereas TPEF emission might occur at ~ 500 nm for the same target excited at 810 nm. Diagram is reproduced and modified with kind permission from Pantazis *et al.*,⁸³ with acknowledgment to National Academy of Sciences, USA.

FIGURE 2.

Second harmonic generation is a coherent process. Because SHG is a coherent process, in this cartoon we illustrate how at least four aspects of collagen's homeostasis may influence collagen's raw TPM SHG signal, an antibody Stain for quantifying collagen levels, and the ratio of forward-scattered to backward-scattered SHG (F/B) for collagen. SHG is sensitive to the regular ordering of scatterers, so conversion of disordered collagen triple helices into ordered fibrils, or de novo synthesis/breakdown of ordered fibrils can alter the detected SHG. Compaction of fibrils can also alter the detected SHG by changing the spacing of fibrils and hence altering interference properties. Lastly, the diameter of ordered fibrils dictates the F/B ratio for collagen SHG.⁴⁴ Readers should appreciate that this overview, simplified for pictorial presentation and clarity, does not reflect all possible signal changes. For example, organization or compaction changes could alter antibody stain intensity, depending on how antibody binding epitopes get rearranged.

FIGURE 3.

Tumor collagen produces strong SHG. Collagen in an *ex vivo* E0771 mammary tumor produces a strong SHG signal in response to near-IR two-photon irradiation, showing the fibrous structure characteristic of highly ordered collagen fibers. All work was approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC).

FIGURE 4.

Quantifying tumor collagen SHG. (a) Collagen in an *ex vivo* E0771 mammary tumor can be immunohistochemically (IHC) labeled and quantified with an anti-collagen antibody (pseudocolored red). (b) Collagen produces a strong SHG signal (pseudocolored red) (same field as in (a)). (c) Dividing the SHG (b) image signal by the IHC (a) image signal (SHG/IHC) provides a new “ratiometric” image (c) which indicates levels of SHG relative to total collagen on a pixel-by-pixel basis. Hence circled regions indicate where this ratio is clearly different (i.e., high or low pixel values), suggesting differences in relative collagen organization in these regions. Increased SHG signal relative to immunolabeling may represent a conversion of disordered triple helices to ordered fibrils, or compaction of fibrils.⁵¹ An increase in both signals may represent *de novo* synthesis of ordered triple helices. Green signal is a nuclear counterstain. All work was approved by and performed in accordance with the guidelines of the IACUC. Diagram is modified from Brown *et al.*,⁸ with permission, and acknowledgment to Nature Publishing Group.