Title:

Polarization-dependent second-harmonic generation for collagen-based differentiation of breast cancer samples

Authors:

Vassilis Tsafas, Evangelia Gavgiotaki, Maria Tzardi, Effrosyni Tsafa, Costas Fotakis, Irene Athanassakis, George Filippidis

Manuscript

The original publication may be found at:

Journal: Journal of Biophotonics 13, 10, e202000180 (2020)

DOI: <https://doi.org/10.1002/jbio.202000180>

Full Article**Polarization-dependent second-harmonic generation for collagen-based differentiation of breast cancer samples**

Vassilis Tsafas^{1,2}, Evangelia Gavgiotaki^{1,3}, Maria Tzardi³, Effrosyni Tsafa¹, Costas Fotakis¹, Irene Athanassakis^{4,*}, George Filippidis^{1,*}

¹ Institute of Electronic Structure and Laser, Foundation for Research and Technology, Heraklion 71110, Crete, Greece

² Department of Physics, University of Crete, 71003 Heraklion, Crete, Greece

³ Medical School, University of Crete, Heraklion 70013, Crete, Greece

⁴ Biology Department, University of Crete, Heraklion 70013, Crete, Greece

***Correspondence:**

Prof. Irene Athanassakis, Department of Biology, University of Crete, University Campus, Heraklion 70013, Crete, Greece

Email: athanire@uoc.gr

Dr. George Filippidis, Institute of Electronic Structure and Laser, Foundation for Research and Technology, Heraklion 71110, Crete, Greece

Email: filip@iesl.forth.gr

KEYWORDS: non-linear optical microscopy, second harmonic generation imaging, polarization-dependent measurements, collagen, breast cancer, diagnosis

Abstract

Non-linear optical imaging techniques have been widely used to reveal biological structures for accurate diagnosis at the cellular as well as the tissue level. In the present study, polarization-dependent second-harmonic generation (PSHG) was used to determine collagen orientation in breast cancer biopsy tissues (grades 0, I, II and III). The obtained data were processed using Fast Fourier Transform (FFT) analysis, while SHG anisotropy and the "ratio parameter" values were also calculated. Such measurements were shown to be able to distinguish collagen structure modifications in different cancer grades tested. The analysis presented herein suggests that PSHG imaging could provide a quantitative evaluation of the tumor state and the distinction of malignant from benign breast tissues. The obtained results also allowed the development of a biophysical model, which can explain the aforementioned differentiations and is in agreement with the simulations relating the SHG anisotropy values with the mechanical tension applied to the collagen during cancer progression. The current approach could be a step forward for the development of new, non destructive, label free optical diagnostic tools for cancer reducing the need of recalls and unnecessary biopsies, while potentially improving cancer detection rates.

1 INTRODUCTION

Non-linear imaging microscopy modalities (multi-photon excitation fluorescence (MPEF), second and third-harmonic generation (SHG, THG)) comprise powerful tools for biomedical diagnosis. These technologies provide several advantages compared to other optical techniques such as high resolution, label-free imaging capabilities, increased penetration depths and intrinsic three dimensional sectioning, while the phototoxicity phenomena onto the biological sample are minimized. Several reports have provided important information for both cells and tissues during inflammation and cancer upon application of these non destructive techniques[1–4]. A previous study from our group demonstrated that THG could evaluate the density and size of tumor cells within malignant tissues and suggested the use of such technology in cancer prognosis[5]. Recently, this technology has been applied on live tissue with very promising results and has therefore been proposed for clinical studies[6,7].

Collagen, which has been considered as cancer biomarker[8–11], is an ideal emitter of high SHG signals due to its non-centrosymmetric structure[12]. Indeed collagen has been considered as a double-edged sword in tumor progression, playing a determinant role, initially in protecting against and later in facilitating tumor cell migration and cancer progression[13]. Collagen is a major structural protein of the extracellular matrix (ECM) consisting of one or multiple fibrils, each one made of three polypeptide α -chains, held together with hydrogen bonds to form a collagen helix[14].

During tumor progression, the ECM undergoes remodeling, characterized by collagen re-deposition and cross-linking, which under the biomechanical tension of tumor growth becomes accessible to enzymatic digestion, facilitating thus tumor expansion[15]. Briefly, collagen, being the most abundant and most difficult to penetrate component of ECM, when remodeled in response to tumor expansion, increases in density and consequently its mechanical strength protects from further tumor expansion[16,17]. Thickened collagen fibers inhibit enzymatic access and cleavage. However, extensive tumor expansion exerts traction

forces on collagen causing its deformation until reaching a critical point, leading finally to triple helix separation, and thus collagen becomes susceptible to enzymatic cleavage[15]. These features are more prominent in higher grades of breast cancer. Unwinding and remodeling of collagen leads to the formation of novel focal points for tumor cell migration and proliferation, infiltration of immune cells with tumor inhibitory or stimulatory effects, which, however, in most cases, promote tumor metastasis[15,18]. Therefore, following collagen behavior could provide very important information in the evaluation of cancer progression.

Breast cancer, which is considered as a typical example of collagen fiber remodeling[19], is one of the most common malignancies in women[20]. For that reason, the efficient treatment of patients, which is based on early and accurate diagnosis, is extremely important. Unfortunately, until now, no ideal markers that could detect all breast cancer cases have been established[20]. Therefore, the development of novel, non-invasive, label free optical techniques that can provide new insights for breast cancer research is of high importance over the last decades[21].

The dependence of non-linear signal from the incident polarization of the excitation radiation can provide additional quantitative information about the samples even at sub-cellular level[22–24]. This information is relevant with the orientation of sample's SHG emitters at molecular and supramolecular scale. At molecular level and for cylindrical symmetry molecules, polarization-dependent SHG (PSHG) can provide the angle between SHG emitters and their molecular symmetry axis. In case of collagen, myosin and starch samples, this angle is in perfect agreement with the helix angle of the specimens[25–27]. At supramolecular scale the corresponding information is gained by the sample fibers' direction using a laboratory coordinated system[28,29]. PSHG imaging has also been used to characterize the molecular architecture of main endogenous sources of contrast in tissues[30–32].

In the present study, the non-linear imaging modality of PSHG was employed as a tool for breast cancer diagnosis. Specifically, quantitative information about structure and directionality of SHG emitters, as represented by collagen, within thin breast tissue sections was obtained upon acquisition of PSHG measurements, which could provide information on the differentiation of benign from cancerous breast tissues. The biophysical model proposed herein could be used for the development of an accurate objective diagnostic tool for breast cancer, based on the PSHG information arising from collagen remodeling.

2 Materials and Methods

2.1 Biological samples

Benign and cancerous tissues were obtained from 8 patients, including 2 benign bearing tumor cases (grade 0, ductal carcinoma), 2 patients with grade I tumors, 2 patients with grade II tumors and 2 patients with grade III tumors. All tissues tested herein were provided from the University Hospital at Heraklion, Crete. The experimental protocol was approved by the Ethics and Scientific Committees of the Hospital (IDs 16/16-10-2019 (579) and 4428/10-07-2019; Crete, Greece). The samples were taken from patients older than 60 years.

Tissue samples were embedded in paraffin and were sliced in 5-10 μm thickness for histological sections and then routinely were stained with hematoxylin and eosin (H&E) for optical microscopy examination[33]. For non-linear imaging, unstained tissue sections of 5-10 μm thickness were placed on special very thin (0.07mm) round glass microscope slides of 3.5cm diameter to provide a flat sample surface. In addition, H&E images of serial sections of the same sample were recorded for the evaluation and comparison with the non-linear data. A recent study has reported the feasibility to record high SHG signals from paraffin-embedded tissue sections[34].

Experienced pathologists had reviewed H&E slides prior to SHG imaging, ensuring accuracy of the overall grade (0, I, II and III). Small samples, less than 1 cm² were investigated. In this study the selection of scanning regions for collecting non-linear images was accomplished by looking in parallel through a light microscope, the corresponding H&E stained images. Multiple regions of each tissue, at least 10, were scanned.

2.2 PSHG Set up

In this study, SHG signals were collected from a custom-made experimental setup, which has been previously described in detail[5,35]. Briefly, the experimental apparatus consisted of a femtosecond fiber based laser oscillator (FemtoPower 1060-5-150-s, 1064 nm, 80 MHz, 4 W, 150 fs, Fianium Ltd) and a modified Nikon upright microscope (Nikon Eclipse MED600D). The energy per pulse at the sample plane was 1 nJ. An objective lens (Carl Zeiss) with moderate numerical aperture (NA) of 0.8 was used to focus the laser beam onto the sample. The scanning procedure (xy direction) was performed with a pair of silver coated galvanometric mirrors (Cambridge Tech. 6210H). The samples were fitted into a motorized xyz translation stage (Standa 8MT167-100) and the focal plane was selected with this stage (1 μ m resolution). Lab View interface controlled both scanning and data acquisition procedures. SHG signals were collected in forward direction using a photomultiplier tube (PMT Hamamatsu H9305-04) and a second objective lens. A bandpass interference filter (Thorlabs 532-10) and a short pass filter (Semrock 720) were placed at the PMT input to cut off the transmitted laser light and solely detected SHG signals from the samples. Typical time duration for obtaining a 2-D 500x500 pixels (125x125 μ m) SHG image was one second. To improve the signal to noise ratio (SNR), 3 scans were realized for each image. A zero-order half-wave plate (WPH05ME-1064, Thorlabs) was placed before galvanometric mirrors for manually rotation of the laser's linear polarization onto the sample plane, in order to create

PSHG data sets that used in our analysis. For compensating the polarization ellipticity, when a dichroic mirror is being employed, a quarter wave plate is advisable to be introduced in the optical path[36]. However in our case, silver coating mirrors were used to minimize the induced polarization ellipticity at the sample plane. The extinction ratio, using cross polarization measurements at the sample plane, was measured to be higher than 25:1 for all linear polarization orientations, which is in agreement with a study that employed a similar set-up[37].

2.3 PSHG Theoretical Biophysical model

The mathematical formula that total SHG intensity obeys when the collagen fibrils are well aligned and parallel with fiber axis is given from the following Equation[22,23,25,38]:

$$I_{shg} = E \cdot \{(\sin[2(a - f)])^2 + [B \cdot (\cos(a - f))^2 + (\sin(a - f))^2]^2\} \quad (1)$$

Where E is an overall multiplication factor, f denotes the angle between the initial polarization of the laser and the projection of collagen fiber onto the polarization plane, and α is the rotation angle of the laser linear polarization (resulting from half-wave plate). Furthermore, SHG intensity signals arising from each fibril follow the form of Equation 1, if it is presumed that each fibril consists from well aligned collagen molecules parallel to its axis. The factor B is called anisotropy parameter and is defined as[26]:

$$B = XR \cdot \sin^2(\delta) + 3 \cdot \cos^2(\delta) \quad (2)$$

XR is defined as the ratio $\frac{\chi_{33}}{\chi_{15}}$, where χ_{33} and χ_{15} are the only non-vanishing and independent second order susceptibility tensor elements and can be evaluated by analyzing the polarization of SHG[39]. Samples that present more organized collagen structures lead to the increment of B values [40,41]. This trend is also observed in studies, where anisotropy coefficient β is calculated and measured [42]. Angle δ in Equation 2 corresponds to the angle between laser's propagation and fiber's axes. The above angles appear schematically in Figure 1a. When

$\delta=90^\circ$, from Equation 2 the anisotropy parameter value defined as $B=XR$, and in the case where each fibril is made of triple helices parallel to the fibril axis, B gives the effective angle θ_e which is the most probable angle between peptide bond and principle axis of fibril[27,43], as it is shown in Figure 1b. The next equation describes this effective angle θ_e :

$$\cos^2(\theta_e) = \frac{XR}{2+XR} \quad (3)$$

In the case of collagen, effective angle θ_e matches with the pitch angle of its helices[27]. In order to reduce the computational time, Equation 1 can be transformed to the following mathematical form [31]:

$$I_{SHG} = c_0 + c_1 \cdot \cos(2 \cdot (a - f)) + c_2 \cdot \cos(4 \cdot (a - f)) \quad (4)$$

Where,

$$B = \sqrt{\frac{c_0+c_1+c_2}{c_0-c_1+c_2}} \quad (5)$$

The advantage of the above approach is that it makes feasible to calculate c_0 , c_1 , c_2 coefficients (therefore B) and the f angle by applying FFT (Fast Fourier Transform) data processing for several different polarization angles a [31,44]. The computational time of FFT for 500x500 pixels images is 10^4 orders of magnitude faster as compared with the utilization of a non-linear fitting algorithm for the Equation 1.

2.4 PSHG Data analysis

The analysis for obtaining the anisotropy parameter B values for each pixel of a SHG image is described below. A 3D matrix (PSHG matrix) was produced from eighteen SHG images of the same area, rotating each time the laser's linear polarization by ten degrees ($\alpha=0^\circ-170^\circ$). The first two dimensions of the PSHG matrix refer the location of the pixels from a SHG image, while the third dimension to the value of each pixel for all laser polarizations. Afterwards, the coefficients c of Equation 4 and through them the anisotropy parameter B can be calculated by applying FFT along the third dimension of the PSHG matrix. By integrating

the total amount of image pixels, the mean anisotropy parameter ($\langle B \rangle$) can be extracted. Erroneous pixels, like noise pixels, which are not in line with the model, must be excluded. In this study, as a filtering criterion, a threshold value was used as referred in literature[29] for the determination the coefficient R^2 , and pixels below this value were excluded from further processing. Specifically, the threshold value of 0.90 was set, in order to verify that the results fit into the model and at the same time, to ensure a high enough number of pixels for the statistical analysis.

Figure 2 presents a characteristic example of PSHG data analysis of a benign tissue specimen. The mean value of B ($\langle B \rangle$) deriving from pixels with $R^2 > 0.9$ is 1.742 ± 0.001 (S.E.M.). Moreover, in the framework of the current study a new factor called "ratio parameter" was introduced for providing additional valuable, quantitative information through PSHG analysis of the collected data. The value of this parameter was defined as the number of collagen pixels with $R^2 > 0.9$ divided by the total number of collagen pixels. As countable collagen pixels were considered only these that presented minimum value along the third dimension of the PSHG matrix over the system noise. For the tissue analyzed in Figure 2, this ratio parameter was equal to 0.388. The above data analysis was performed through a specially constructed algorithm that was designed and programmed in a MATLAB environment for analyzing PSHG data sets and extracting for each area the mean B as well as the ratio parameter values.

2.5 Statistical analysis

For multi group comparisons, two-tailed unpaired Student's T-test was applied. P-values < 0.05 were considered significant (*), values < 0.01 were considered very significant (**), and values < 0.001 and < 0.0001 were considered highly significant (***) and ****). Statistics were performed using GraphPad Prism 6.01 (Graphpad Software, La Jolla, CA).

3 Results

In an effort to investigate the potential collagen's directionality and organization modifications during the progression of breast cancer without the need of staining, it was attempted to visualize and obtain qualitative information from collagen structures in thin sections of breast tissue by collecting SHG signals.

Serial sections of breast tissue biopsies were submitted to H&E staining as well as SHG imaging (Figure 3). Such manipulation allowed examination of H&E images staining of benign and malignant breast tissues and the corresponding non-linear unstained microscopy images (SHG), so that monitoring reflects the same area of the sample. SHG measurements correspond to collagen organization and distribution within the tissue (Figure 3a). As described in the histology-based literature collagen surrounding normal epithelial structures are curly and smooth, while in tumors collagen develops in parallel with the oncogenic tissue, progressively thickens, linearizes and stiffens[19]. Such description completely fitted the SHG-derived images, where thicker and linearized collagen could be visualized around the carcinoma, without the need of use of any histological staining (Figure 3a).

To further characterize the cancerous areas within breast tissue biopsies, PSHG data analysis, as described in materials and methods section, was applied to different cases of breast tumor severity ranging from benign grade 0 to grades I, II and III. In all cases, the scanning regions studied were limited to 125 μm x 125 μm dimensions. Upon acquisition of PSHG images of all tissue types tested, PSHG analysis as to the characteristic anisotropy and ratio parameter values was performed (Figure 4).

The application of PSHG to grade 0 tumor samples (Figure 4a) nicely showed the thickening linearization of collagen, which also showed the lowest anisotropy factor B (Figure 4e). Tumor severity is accompanied with collagen remodeling, likely corresponding to the

collagen susceptibility to enzymatic cleavage[15], which is accompanied by increasing B values and decreasing ratio parameter (Figure 4 b-d and f-h).

Collecting the PSHG data sets from eight patients in grades 0, I, II and III, the mean values of anisotropy parameter $\langle B \rangle$ as well as the mean ratio parameters were calculated as described in the section of materials and methods (Figure 5).

Thus, the anisotropy parameter $\langle B \rangle$ showed a significant increase of $\langle B \rangle$ value until grade II tumor progression. Grades II and III did not show any statistically significant difference in $\langle B \rangle$ values. Apparently, in the grade II and III samples analyzed herein, collagen could have been disrupted by enzymatic cleavage, showing at the same time an increased anisotropy $\langle B \rangle$ value, which, however, could not distinguish among the two different grades (Figure 5a). In contrast, the mean ratio parameter values showed a highly significant decrease between grade II and III stages (Figure 5b).

Thus, if $\langle B \rangle$ values were affected by collagen disruption and fibril orientation[45], this could mean that not all fibrils were completely parallel to fiber axis, which resulted in an anisotropy reduction of the fiber. For fully isotropic arrangement of fibrils the expected value of the fiber anisotropy parameter is close to the unit, while increasing along with the alignment of the fibrils across the fiber axis[40,41]. For verifying this hypothesis, the effects of angles f and δ orientation disorders on the calculated from the PSHG analysis anisotropy parameter B values were simulated. This procedure takes into account the contribution of each collagen fibril intensity value within the focal volume for the calculation of the total SHG intensity[41,46]. Considering that the f and δ angles have constant mean values and follow normal distributions around themselves, with the same standard deviation σ , meaning that standard deviation σ represents the orientation disorders of f and δ angles, B values tend to the unit as σ increases (Figure 6a, simulation from Equation 1,4 and 5).

As an example, considering that $\theta=45^\circ$ in Equation 3, which corresponds to the expected collagen helix angle as evaluated by X-ray diffraction experiments[27], a theoretical value of B in the order of 2 ($B_{th}=2$) is obtained (Figure 6a). The calculated B values tend to the unit when σ increases, and this trend is independent of B_{th} [41]. At the same time for $\sigma<20^\circ$, R^2 remains close to the unit (Figure 6b). Therefore, up to that point, PSHG data seem to fit perfectly to the biophysical model. Such analysis implies that the reduction of the ratio parameter values at higher grades of tumor progression, observed in Figure 5b, could not be attributed to the f , δ angle orientation disorders, indicating that different morphological modifications of the collagen structure occur after the grade II scoring. These theoretical results were in perfect agreement with the biological events, where after tumor expansion reaches a critical point, collagen is deformed by triple helix separation, becoming susceptible to enzymatic cleavage, forming thus novel focal points for tumor cell development and disease aggravation.

4 Discussion

In an effort to quantitatively correlate PSHG imaging to differentiate benign from breast cancer samples, tissue biopsies from cancer grades 0, I, II and III were submitted to PSHG imaging and data were then processed using Fast Fourier Transform analysis, while also calculating second-harmonic anisotropy and the "ratio parameter" values. The obtained results indicated that PSHG data analysis could correlate the increment of the anisotropy parameter B (Figure 5a) with tumor progression[30]. Such behavior could indeed be due to the mechanical tensions applied to collagen during cancer progression.

The orientation disorders of fibrils result in the reduction of the anisotropy parameter of the fiber as predicted by the theoretical calculations in Figure 6a. In the case of collagen, it is assumed that the mechanical stretch further aligns the fibrils with the fiber axis, reducing thus

the standard deviation σ of angles f and δ (Figure 7a and 7b) and finally increasing anisotropy parameter B values. This is in accordance with the measured values of B presented in Figure 5a. Furthermore, mechanical stretching may decrease collagen helix angle as shown in Figure 7c and 7d. This reduction of collagen helix angle, according to Equation 3 in the section of materials and methods, leads to an increase of ratio XR and consequently to a raise of anisotropy parameter B values, as foreseen from Equation 2 of the same section.

During cancer progression, collagen structure is deformed and enzymatic cleavage results in a crumbled image[15,18] as the one presented in Figure 4d. The observed discontinuities in collagen detected by the SHG signal in Figure 4d (grade III sample) could be due to the triple helix separation, while other collagen areas could be only affected by structural organization modifications. Such changes could be expressed through the quantitative calculation of the ratio parameter values presented in Figure 5b. The reduction of ratio parameter value at grade III could be nicely explained by the breakdown of the collagen's cylindrical symmetric structure. As a consequence, SHG cannot be described by Equation 1, indicating the use of different biophysical model at that point. A recent work demonstrated that a more generalized model, considering both cylindrical and trigonal symmetry, could be used to describe the organization of collagen structure[47]. Such approach could provide improved results in human breast samples[48].

Following the experimental approach described herein, which was based on the calculation of the integrated anisotropy parameter values of different collagen types, the discrimination between benign and malignant cancer tissue samples could be achieved. A study related to the differentiation of biochemically different collagen types, showed that the average anisotropy parameter values in collagen I and II, from tendon and cartilage respectively, were similar[41]. Therefore, the findings of the aforementioned study[41] indicate that the biochemical nature

of collagen does not influence the anisotropy parameter values, which could be considered to be governed by the biomechanical properties of collagen fibers.

It has to be noted that in the experimental design of the current work, a forward detection scheme was employed for the collection of the SHG images from thin paraffin-embedded sections. Although SHG anisotropy parameter values depend on the detection geometry[49], the extracted information could be useful as a novel diagnostic tool for a quick characterization of newly excised thin biopsy samples.. A recent study using PSHG reflection measurements at colon mucosa samples shows that cancer may increase collagen fibrils organization even 20 cm away from cancerous area[42].

It is worth underlining that the applied technology has the ability to also act synergistically with other non-linear processes, such as third-harmonic generation and polarization-dependant THG (PTHG) into a single, unified microscopy platform, providing thus complementary structural information from the different types of tissues. Using a single beam, without the need of staining, except from probing collagen modifications via PSHG analysis, information on lipid bodies concentration, inhomogeneities, membranes[4,5,35], as well as ordered lipid assemblies[50] through the performance of THG and PTHG measurements respectively, could be obtained. Thus the unique attributes of non-linear microscopy provide promising imaging modalities as potential new, non invasive tools for disease diagnostics in clinic. Non-linear endoscopes have been already employed for the imaging of thick animal tissues[51,52] and it is anticipated that the integration of polarization-dependant techniques could provide additional unique molecular information.

5 Conclusions

There is ongoing research for the development of new, non destructive, label free optical tools for reducing biopsy recalls while maintaining or improving cancer detection rates. The

findings presented herein suggest that PSHG measurements based on quantitative criteria (B and ratio parameter values) could evaluate the tumor state, differentiate malignant from benign breast tissue samples and distinguish the different grades of cancer. Specifically, the obtained PSHG data showed increased calculated second-harmonic anisotropy parameter values, which is an indication for collagen structure organisation in tumor progression. Moreover, the proposed biophysical model that was verified via simulation analysis, correlated SHG anisotropy values with the mechanical tension applied to collagen during cancer progression. Although in the framework of the current study breast cancer tissues were investigated, it is anticipated that PSHG modality can be used as an innovative diagnostic tool for the early diagnosis of inflammation in various diseases.

This study has a significant clinical potential, since it can monitor quantitative changes in the sub-cellular behavior of collagen in different types of breast human tissues. However, further studies are needed for the application of this very promising technique in the clinical environment.

ACKNOWLEDGMENTS

The present work was supported by “LASERLAB EUROPE V” (871124). V. Tsafas acknowledges the financial support from General Secretariat for Research and Technology (GSRT) and Hellenic Foundation for Research and Innovation (HRFI) for the financial support.

AUTHOR CONTRIBUTIONS

In the present study, V.T. performed the experiments and was assisted by E.G. All biological samples were prepared by M.T. V.T. was responsible for the data analysis. C.F. was involved

in the conception of the work. G.F. and I.A. conceived and supervised the project. V.T., E.G., E.T., I.A. and G.F. wrote the paper.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

- [1] K. Harpel, R. D. Baker, B. Amirsolaimani, S. Mehravar, J. Vagner, T. O. Matsunaga, B. Banerjee and K. Kieu, *Biomed. Opt. Express*, **2016**, 7, 2849.
- [2] K. Palikaras, M. Mari, B. Petanidou, A. Pasparaki, G. Filippidis and A. N. Tavernarakis, *J. Lipid Res.*, **2017**, 58, 72.
- [3] M. G. Giacomelli, T. Yoshitake, L. C. Cahill, H. Vardeh, L. M. Quintana, B. E. Faulkner-Jones, J. Brooker, J. L. Connolly and J. G. Fujimoto, *Biomed. Opt. Express*, **2018**, 9, 2457.
- [4] E. Gavgiotaki, G. Filippidis, H. Markomanolaki, G. Kenanakis, S. Agelaki, V. Georgoulas and I. Athanassakis, *J. Biophotonics*, **2017**, 10, 1152.
- [5] E. Gavgiotaki, V. Tsafas, S. Bovasianos, S. Agelaki, V. Georgoulas, M. Tzardi, I. Athanassakis and G. Filippidis, in *Advances in Microscopic Imaging II*, eds. F. S. Pavone, E. Beaurepaire and P. T. So, SPIE, **2019**, 11076, 18.
- [6] S. You, H. Tu, E. J. Chaney, Y. Sun, Y. Zhao, A. J. Bower, Y. Z. Liu, M. Marjanovic, S. Sinha, Y. Pu and S. A. Boppart, *Nat. Commun.*, **2018**, 9, 1.

- [7] D. Tokarz, R. Cisek, M. N. Wein, R. Turcotte, C. Haase, S.-C. A. Yeh, S. Bharadwaj, A. P. Raphael, H. Paudel, C. Alt, T.-M. Liu, H. M. Kronenberg and C. P. Lin, *PLoS One*, **2017**, *12*, 0186846.
- [8] T. Hompland, A. Erikson, M. Lindgren, T. Lindmo and C. de Lange Davies, *J. Biomed. Opt.*, **2008**, *13*, 054050.
- [9] N. D. Kirkpatrick, M. A. Brewer and U. Utzinger, *Cancer Epidemiol. Biomarkers Prev.*, **2007**, *16*, 2048.
- [10] C. Morrison, J. Thornhill and E. Gaffney, *Urol. Res.*, **2000**, *28*, 304.
- [11] J. Smolle, M. Fiebiger, R. Hofmann-Wellenhof and H. Kerl, *Am. J. Dermatopathol.*, **1996**, *18*, 358.
- [12] P. J. Campagnola and L. M. Loew, *Nat. Biotechnol.*, **2003**, *21*, 1356.
- [13] M. Fang, J. Yuan, C. Peng and Y. Li, *Tumor Biol.*, **2014**, *35*, 2871.
- [14] B. Brodsky and A. V. Persikov, *Adv. Protein Chem.*, **2005**, *70*, 301.
- [15] P. A. Torzilli, J. W. Bourne, T. Cigler and C. T. Vincent, *Semin. Cancer Biol.*, **2012**, *22*, 385.
- [16] P. P. Provenzano, K. W. Eliceiri, J. M. Campbell, D. R. Inman, J. G. White and P. J. Keely, *BMC Med.*, **2006**, *4*, 38.
- [17] X. Tang, T. B. Kuhlenschmidt, J. Zhou, P. Bell, F. Wang, M. S. Kuhlenschmidt and T. A. Saif, *Biophys. J.*, **2010**, *99*, 2460.
- [18] L. Bingle, N. J. Brown and C. E. Lewis, *J. Pathol.*, **2002**, *196*, 254.
- [19] J. N. Wolfe, *Cancer*, **1976**, *37*, 2486.
- [20] A. M. Kabel, *J. Oncol. Sci.*, **2017**, *3*, 5.
- [21] S. K. Paidi, A. Rizwan, C. Zheng, M. Cheng, K. Glunde and I. Barman, *Cancer Res.*, **2017**, *77*, 247.
- [22] W. L. Chen, T. H. Li, P. J. Su, C. K. Chou, P. T. Fwu, S. J. Lin, D. Kim, P. T. C. So

- and C. Y. Dong, *Appl. Phys. Lett.*, **2009**, *94*, 183902.
- [23] S. W. Chu, S. Y. Chen, G. W. Chern, T. H. Tsai, Y. C. Chen, B. L. Lin and C. K. Sun, *Biophys. J.*, **2004**, *86*, 3914.
- [24] M. Pinsard, S. Laverty, H. Richard, J. Dubuc, M. C. Schanne-Klein and F. Lgar, *Sci. Rep.*, **2019**, *9*, 1.
- [25] S. V. Plotnikov, A. C. Millard, P. J. Campagnola and W. A. Mohler, *Biophys. J.*, **2006**, *90*, 693.
- [26] Z. Y. Zhuo, C. S. Liao, C. H. Huang, J. Y. Yu, Y. Y. Tzeng, W. Lo, C. Y. Dong, H. C. Chui, Y. C. Huang, H. M. Lai and S. W. Chu, *J. Struct. Biol.*, **2010**, *171*, 88.
- [27] F. Tiaho, G. Recher and D. Roude, *Opt. Express*, **2007**, *15*, 12286.
- [28] P. Stoller, K. M. Reiser, P. M. Celliers and A. M. Rubenchik, *Biophys. J.*, **2002**, *82*, 3330.
- [29] G. Ducourthial, J. S. Affagard, M. Schmeltz, X. Solinas, M. Lopez-Poncelas, C. Bonod-Bidaud, R. Rubio-Amador, F. Ruggiero, J. M. Allain, E. Beaurepaire and M. C. Schanne-Klein, *J. Biophotonics*, **2019**, *12*, 201800336.
- [30] K. R. Campbell, R. Chaudhary, J. M. Handel, M. S. Patankar and P. J. Campagnola, *J. Biomed. Opt.*, **2018**, *23*, 1.
- [31] I. Amat-Roldan, S. Psilodimitrakopoulos, P. Loza-Alvarez and D. Artigas, *Opt. Express*, **2010**, *18*, 17209.
- [32] D. Roude, E. Schaub, J. J. Bellanger, F. Ezan, J. C. Scimeca, G. Baffet and F. Tiaho, *Sci. Rep.*, **2017**, *7*, 1.
- [33] A. H. Fischer, K. A. Jacobson, J. Rose and R. Zeller, *Cold Spring Harb. Protoc.*, **2008**, *3*, 4986.
- [34] M. G. Monaghan, S. Kroll, S. Y. Brucker and K. Schenke-Layland, *Tissue Eng. - Part C Methods*, **2016**, *22*, 517.

- [35] E. Gavgiotaki, G. Filippidis, I. Zerva, G. Kenanakis, E. Archontakis, S. Agelaki, V. Georgoulas and I. Athanassakis, *J. Biophotonics*, **2019**, *12*, 201800277.
- [36] C.-K. Chou, W.-L. Chen, P. T. Fwu, S.-J. Lin, H.-S. Lee and C.-Y. Dong, *J. Biomed. Opt.*, **2008**, *13*, 014005.
- [37] S. Psilodimitrakopoulos, L. Mouchliadis, I. Paradisanos, A. Lemonis, G. Kioseoglou and E. Stratakis, *Light Sci. Appl.*, **2018**, *7*, 18005.
- [38] R. M. Williams, W. R. Zipfel and W. W. Webb, *Biophys. J.*, **2005**, *88*, 1377.
- [39] A. Erikson, J. Örtengren, T. Hompland, C. de Lange Davies and M. Lindgren, *J. Biomed. Opt.*, **2007**, *12*, 044002.
- [40] A. S. Quigley, S. Bancelin, D. Deska-Gauthier, F. Légaré, L. Kreplak and S. P. Veres, *Sci. Rep.*, **2018**, *8*, 1.
- [41] E. I. Romijn, A. Finnøy and M. B. Lilledahl, *J. Biophotonics*, **2019**, *12*, 201800090.
- [42] S. Z. Despotović, Đ. N. Milićević, A. J. Krmpot, A. M. Pavlović, V. D. Živanović, Z. Krivokapić, V. B. Pavlović, S. Lević, G. Nikolić and M. D. Rabasović, *Sci. Rep.*, **2020**, *10*, 1.
- [43] L. Mostaço-Guidolin, N. L. Rosin and T. L. Hackett, *Int. J. Mol. Sci.*, **2017**, *18*, 1772.
- [44] P. Matteini, F. Ratto, F. Rossi, R. Cicchi, C. Stringari, D. Kapsokalyvas, F. S. Pavone and R. Pini, *Opt. Express*, **2009**, *17*, 4868.
- [45] D. J. S. Hulmes, in *Journal of Structural Biology*, Academic Press Inc., **2002**, *137*, 2.
- [46] F. J. Ávila, O. Del Barco and J. M. Bueno, *J. Biomed. Opt.*, **2015**, *20*, 86001.
- [47] R. Mercatelli, S. Mattana, L. Capozzoli, F. Ratto, F. Rossi, R. Pini, D. Fioretto, F. S. Pavone, S. Caponi and R. Cicchi, *Commun. Biol.*, **2019**, *2*, 1.
- [48] R. Mercatelli, T. Triulzi, F. S. Pavone, R. Orlandi and R. Cicchi, *J. Biophotonics*, **2020**, 202000159.
- [49] C. Teulon, I. Gusachenko, G. Latour and M.-C. Schanne-Klein, *Opt. Express*, **2015**, *23*,

9313.

- [50] M. Zimmerley, P. Mahou, D. Débarre, M. C. Schanne-Klein and E. Beaurepaire, *Phys. Rev. X*, **2013**, 3, 011002.
- [51] G. Ducourthial, P. Leclerc, T. Mansuryan, M. Fabert, J. Brevier, R. Habert, F. Braud, R. Batrin, C. Vever-Bizet, G. Bourg-Heckly, L. Thiberville, A. Druilhe, A. Kudlinski and F. Louradour, *Sci. Rep.*, **2015**, 5, 1.
- [52] A. Lombardini, V. Mytskaniuk, S. Sivankutty, E. R. Andresen, X. Chen, J. Wenger, M. Fabert, N. Joly, F. Louradour, A. Kudlinski and H. Rigneault, *Light Sci. Appl.*, **2018**, 7, 1.
- [53] D. F. Holmes, Y. Lu, T. Starborg and K. E. Kadler, in *Current Topics in Developmental Biology*, Academic Press Inc., **2018**, 130, 107.
- [54] M. J. Buehler, *Proc. Natl. Acad. Sci. U. S. A.*, **2006**, 103, 12285.

FIGURES

FIGURE 1. Schematic view of macroscopic (a) and microscopic (b) biophysical model for PSHG. (a) Collagen fiber (green cylinder) is consisting of fibrils (blue cylinders) which are radiated by laser. The yellow arrow represents the direction of laser propagation at the laboratory's coordinates system (xyz) and δ is the angle between the fiber axis and the laser's propagation direction. Red arrows represent the laser polarizations, which are onto the xy plane. E_{initial} is the initial electric field and E_i the electric field after an α angle of rotation. Angle ϕ is the angle between the initial polarization and the projection of the fiber axis onto the xy plane. (b) Hierarchical structure of collagen fiber (green cylinder) is consisting of fibrils (blue cylinder) which are made of triple helices (black dots). θ represents the angle between the tangent line at any point of the collagen triple helix and the helix axis, which is a good approximation of the radiated electrons' oscillation direction[27,43].

FIGURE 2. Example of FFT analysis of PSHG data from a benign breast tissue sample. a) Full set of PSHG data consist of 18 SHG images from the same area of the tissue. Each time angle α of Equation 4 is rotating by 10° . b) Results of FFT analysis of PSHG data set (a). Each pixel of Figure 2b corresponds to a B value, which was calculated through the determination of c coefficients via FFT analysis and Equation 5. Pixels with $R^2 < 0.9$ were excluded from further analysis and were presented as black spots

FIGURE 3. a) SHG image of ductal carcinoma grade I. The image is 1mm x 1mm in size and consists of 100 sequential sub-images (100μm x 100μm). b) The corresponding H&E stained tissue obtained with a brightfield microscope. The obtained data were recorded from different sequential tissue sections.

FIGURE 4. Representative examples of FFT analysis for benign (grade 0), grade I, grade II and grade III breast tissues. (a-d): The sum of 18 different polarization SHG images for each grade. (e-h): Results of the FFT analysis for each sample a-d.

FIGURE 5. a) Each column represents the mean value of $\langle B \rangle$ (N=20, median values) and error bars indicate the Standard Error of the Mean (S.E.M.) of each grade. b) Each column represents the mean ratio parameter (N=20, median values) and error bars indicate the S.E.M. of each grade. ns: no significant, ** and **** statistically significant (<0.01 and <0.0001 respectively).

FIGURE 6. Theoretical effects of angles f and δ disorders (σ) to anisotropy parameter B (a) and R^2 (b) values. In both cases it is assumed that the focal volume is $\sim 3 \mu\text{m}^3$ and includes 100 fibrils with mean diameter and length in the order of 200 nm and 1 μm respectively[53,54]. This is considered to be a good approximation for the focusing conditions that were employed (N.A.=0.8). Each fibril follows Equation 1 with $B_{\text{th}}=2$ and angles f and δ following a normal distribution with the same standard deviation σ and mean values 0° for f and 90° for δ . For each σ , 10 different PSHG data sets are

generated, and PSHG analysis was applied each time for the calculation of anisotropy parameter B and R^2 values. Error bars corresponds to S.E.M.

FIGURE 7. Schematic view of collagen's fiber, fibril and helix before (a,c) and after (b,d) mechanical tension. In the case of mechanical tension collagen fibrils are almost parallel to the fiber and the angle of the helix decreases ($\theta_2 < \theta_1$). The scales of the figure in the cartoon are arbitrary.

Graphical Abstract

The development of label free non invasive techniques to be used as novel diagnostic tools in cancer research is of great importance for improving the quality of life for millions of patients. Information concerning the structure and directionality of collagen fibers in thin breast tissue sections was collected using polarization-dependent SHG imaging measurements. This approach enables to quantitatively differentiate benign from cancerous breast biopsy samples thus PSHG could be a unique tool helping to improve cancer detection rates.